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Modification and Uptake of a Cisplatin Carbonato Complex by Jurkat Cells[S]

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ABSTRACT

The interactions of Jurkat cells with cisplatin, *cis*-[Pt($^{15}NH_3$)$_2$Cl$_2$] (**1**), are studied using ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) NMR and inductively coupled plasma mass spectrometry. We show that Jurkat cells in culture rapidly modify the monocarbonato complex *cis*-[Pt($^{15}NH_3$)$_2$(CO_3)Cl]$^-$ (**4**), a cisplatin species that forms in culture media and probably also in blood. Analysis of the HSQC NMR peak intensity for **4** in the presence of different numbers of Jurkat cells reveals that each cell is capable of modifying 0.0028 pmol of **4** within ~0.6 h. The amounts of platinum taken up by the cell, weakly bound to the cell surface, remaining in the culture medium, and bound to genomic DNA were measured as functions of time of exposure to different concentrations of drug. The results show that most of the **4** that has been modified by the cells remains in the culture medium as a substance of molecular mass <3 kDa, which is HSQC NMR silent, and is not taken up by the cell. These results are consistent with a hitherto undocumented extracellular detoxification mechanism in which the cells rapidly modify **4**, which is present in the culture medium, so it cannot bind to the cell. Because there is only a slow decrease in the amount of unmodified **4** remaining in the culture medium after 1 h, -1.1 ± 0.4 μM h^{-1}, the cells subsequently lose their ability to modify **4**. These observations have important implications for the mechanism of action of cisplatin.

The anticancer drug cisplatin, *cis*-[Pt(NH_3)$_2$Cl$_2$] (**1**; Scheme 1) is effective against many different types of cancer (Rosenberg, 1971; Eastman, 1999; Boulikas and Vougiouka, 2004; Wang and Lippard, 2005). The clinical formulation of cisplatin, in 154 mM aqueous NaCl, contains a mixture of species, being mainly **1** with some of the monoaquated species, *cis*-[Pt(NH_3)$_2$Cl(H_2O)]$^+$ (**2**) (Miller and House, 1990). Introduction of the drug into blood, which has a lower chloride concentration (~105 mM), leads to formation of additional **2**. Because the pK_a value for deprotonation of **2** is 6.53 (Miller and House, 1990; Berners-Price and Appleton, 2000), **2** exists mainly in the deprotonated hydroxo form, *cis*-[Pt(NH_3)$_2$Cl(OH)] (**3**) at physiological pH.

Previously (Centerwall et al., 2005), we used ^{15}N-labeled **1** and ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) NMR to show that **3** rapidly reacts with carbonate in culture medium, most likely with dissolved CO_2 (Palmer and van Eldik, 1983; Acharya et al., 2004), to form the carbonato complex, *cis*-[Pt(NH_3)$_2$(CO_3)Cl]$^-$ (**4**; Scheme 1). Because the carbonate concentration in blood and the cytosol is relatively high, ~24 mM, **4** is likely to be present under conditions of therapy. If enough Jurkat cells are present in the culture medium, **4** is not observable in the HSQC NMR spectrum, implying that it is rapidly taken up and/or modified by the cells (Tacka et al., 2004).

In this report, we use ^{1}H-^{15}N HSQC NMR and inductively coupled plasma mass spectrometry (ICP-MS) to study the interaction of cisplatin with Jurkat cells. The NMR studies show that the cells rapidly modify (within ~0.6 h) some or all of **4** present in the culture medium, with modified **4** remaining in the medium incapable of binding to cells. The molecular mass of modified **4** is <3 kDa, and it is not detectable with HSQC NMR. From the measured amounts of unmodified **4** remaining in the medium in the presence of different numbers of cells, we calculate that each cell modifies 0.0028 pmol of this carbonato complex. Continued exposure of the

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ABBREVIATIONS: HSQC, heteronuclear single quantum coherence; ICP-MS, inductively coupled plasma mass spectrometry; [Pt]$_o$, unmodified platinum; nt, nucleotide.

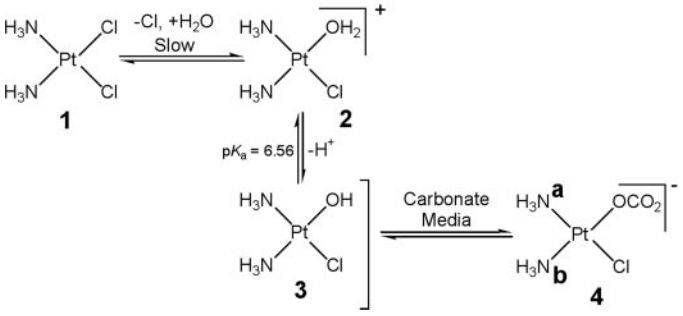
cells to **4** (and **1**) results in uptake of platinum, platination of genomic DNA, and cell death.

In addition to the rate at which **4** disappears from solution, we measure the rate at which platinum becomes weakly attached to cells (removable by washing) and the rate at which platinum becomes strongly bound to and/or enters cells (not removable by washing). We also measure the rate at which platinum binds to nuclear DNA, because most evidence suggests that only platinum bound to nuclear DNA is effective in causing apoptosis. All three rates are proportional to extracellular cisplatin concentration, providing no evidence of saturation. Comparing rates, we find that less than 1% of the strongly bound (not removable) platinum is bound to nuclear DNA.

These results show that Jurkat cells use a previously undocumented defense mechanism to rapidly modify **4** to prevent it from entering the cytosol and reaching the nucleus. However, the defense signal is terminated when platinum, possibly as unmodified **4**, enters the cell. Because the related platinum drug carboplatin can also form a carbonato complex under biological conditions (Di Pasqua et al., 2006), platinum-carbonato species may be responsible for the cytotoxicity of the platinum drugs.

Materials and Methods

^{1}H-^{15}N Heteronuclear Single Quantum Coherence NMR. The details of the ^{1}H-^{15}N HSQC NMR measurements involving ^{15}N-labeled cisplatin were published previously (Tacka et al., 2004). The HSQC NMR spectra were collected in a capped tube at 37°C using a Bruker DRX500 Advance spectrometer (^{15}N; 50.646 MHz) equipped with a 5-mm triple axis probe. Stock solutions of 3.0 mM [^{15}N]cisplatin, containing 154 mM NaCl, were allowed to reach equilibrium at least 24 h before their use in the NMR experiments, referred to as "aging". The NMR samples were prepared by suspending the indicated number of Jurkat cells in 900 μl of RPMI 1640 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2.0 mM L-glutamine in 95%, 5% H_2O/D_2O, pH 7.2, subsequently referred to as "culture medium". 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 RPMI 1640 medium, was 105 mM. The NMR experiments were two-dimensional, ^{1}H-^{15}N, with inverse detection and decoupling during acquisition without spinning the sample. Each experiment was 62 min (48 scans), giving 10^3 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 ~0.6 h after the addition of drug to the cells. 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 ^{1}H 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.



Scheme 1. Structures of the compounds and the route to their formation.

Modification of the Monocarbonato Complex (4) by Jurkat Cells. To determine the approximate molecular mass of the monocarbonato complex that was modified by exposure to Jurkat cells, samples were passed through molecular mass cut-off filters and analyzed for platinum content using ICP-MS. Three samples, each containing 5×10^6 Jurkat cells suspended in 900 μl of culture medium and 20 μl of 3 mM stock cisplatin in 154 mM NaCl (final total platinum concentration, 65 μM), were incubated under standard conditions in a humidified, 37°C, 5% CO_2 atmosphere for 2 h after which time the cells were pelleted by centrifugation for 6 to 10 min at 200*g*. The supernatant from one sample was passed through a 50-kDa filter (Millipore Corporation, Billerica, MA), whereas that from a second sample was passed through a 3-kDa filter. In each case, the filter was washed with 20 μl of deionized water with the wash being combined with the original filtrate to give a total volume of 940 μl. We added 20 μl of water to the supernatant from the third sample, which was not passed through a filter. We added 500 μl of a 70% (v/v) nitric acid/deionized H_2O solution to all three samples, and the resulting mixture was heated at 70°C for 24 h. Before analysis using ICP-MS (ELAN 6100; PerkinElmerSciex Instruments, Boston, MA), the samples were diluted to a final volume of 13 ml by the addition of deionized H_2O. Within experimental error, all three samples yielded the same concentration of platinum, indicating that all of the platinum in the culture medium has a molecular mass of <3 kDa.

The possibility that modification of **4** by Jurkat cells resulted in the loss of the ^{15}N-labeled ammonia molecules from the monocarbonato complex was examined by exposing 15×10^6 Jurkat cells to **1** (65 μM total platinum) in culture medium in a total volume of 920 μl at 37°C. After 1 h, the cells were removed by centrifugation (200*g*), the supernatant was acidified to pH 2 by the addition of 10 M $HClO_4$, and ^{1}H-^{15}N HSQC NMR data were collected on the acidified sample over a period of 3 h. Aside from **1**, no HSQC NMR peaks in the chemical shift range (δ ^{1}H, 0 to 8.5 ppm; δ ^{15}N, +14 to −120 ppm) were observed. Thus, ammonia, detectable as NH_4^+, was not released in the disappearance of **4**. Also undetected in any of the HSQC NMR experiments are peaks with δ ^{15}N of approximately −40 ppm, the presence of which would indicate attack of **4** by a sulfur nucleophile present in the medium. These adducts, with nitrogen *trans* to sulfur, are relatively long lived and are readily observed in reactions of cisplatin with thiols using HSQC NMR (El-Khateeb et al., 1999; Supplemental Data).

Uptake of Platinum by Jurkat Cells. The uptake study used seven samples, each containing 5×10^6 Jurkat cells suspended in 900 μl of culture medium. The number of cells and their viabilities were determined before the start of each experiment by light microscopy using a hemacytometer under standard trypan blue staining conditions (Allison and Ridolpho, 1980). Viabilities at the beginning of each experiment were ~94%, and after a 6-h exposure to 65 μM cisplatin under the conditions described below, they were ~74%. To each cell sample, we added 20 μl of an aged (24-h-old) stock 3.0 mM solution of cisplatin (Sigma-Aldrich, St. Louis, MO), containing 154 mM sodium chloride, to give a final total platinum concentration of 65 μM.

Six of the samples were placed in capped Eppendorf tubes at 37°C, whereas the seventh sample ($t = 0$) was immediately pelleted in a centrifuge for 3 min at 200*g*. After centrifugation, the culture medium was removed from the cell pellet, and 500 μl of the medium was reserved for analysis of its platinum content using ICP-MS. The cell pellet was resuspended in 1 ml of phosphate-buffered saline, and the cells were pelleted by centrifugation. The supernatant was removed, and 500 μl of the wash solution was reserved for analysis of platinum content using ICP-MS. The remaining cell pellet, containing platinum strongly bound to or taken up by the cells, was digested for 24 h at 70°C in 500 μl of 70% (v/v) nitric acid/deionized water to give a

solution that was analyzed for its platinum content. The above-mentioned procedure was carried out each hour using one of the remaining six samples (t = 1–6), and, in a separate set of experiments, the procedure was repeated with a total platinum concentration in the culture medium of 25 μM. Previous work has shown (Tacka et al., 2004) that exposure of Jurkat cells to 25 μM cisplatin for 3 h results in a significant loss in cell viabilities measured 24 h after exposure to drug.

Quantitation of Platinum-DNA Adducts. Ten million Jurkat cells were incubated with various concentrations of cisplatin for 1, 1.5, or 3 h. The source of cisplatin was Platinol, which contains 3.3 mM cisplatin in 154 mM NaCl. After incubation under standard conditions in a humidified, 37°C, 5% CO_2 atmosphere for the appropriate time and platinum concentration, cellular DNA was isolated, and the number of platinum-DNA adducts was determined as described previously (Sadowitz et al., 2002). Numbers of platinum-DNA adducts were calculated assuming 1 pg of platinum per microgram of DNA corresponds to 1759 adducts per 10^6 nucleotides (nt) (using atomic weight of platinum = 195 g/mol and average molecular weight of a nucleotide = 343 g/mol).

Results

HSQC NMR Experiments. A series of ^{1}H-^{15}N HSQC NMR experiments were carried out with ^{15}N-labeled **1** (total platinum concentration, 65 μM) in the culture medium with various numbers of Jurkat cells. In the absence of cells, NMR peaks for **1** and **4** are observed (Centerwall et al., 2005) in the culture medium, as shown in the spectrum of Fig. 1. The peak arising from cisplatin, **1**, at ^{1}H/^{15}N, 4.09/−68.0, overlaps with one of the peaks of the monocarbonato complex, **4a** (nitrogen *trans* to chloride). The second peak for the monocarbonato complex, **4b** (nitrogen *trans* to CO_3^{2-}), occurs at ^{1}H/^{15}N, 3.61/−80.5, in Fig. 1. Because peaks **4a** and **4b** should have equal intensities, the true intensity of peak **1** is the total intensity of the overlapped peak (**1** + **4a**) minus the intensity of **4b**. All peak intensities decrease with time (Tacka et al., 2004). The sum of peak intensities, extrapolated to t = 0, corresponds to the total concentration (65 μM) of all species at t = 0, making it possible to convert peak intensities at any time to concentrations.

Addition of Jurkat cells to the NMR solution causes a decrease in the intensity of the peak for **4b** (Fig. 1), at the earliest time point. Because the decrease occurs within the time required to collect the first NMR data point, ~0.6 h, the cell-induced change in signal intensity is rapid. Cells cause little change in the peak intensity of **1** at this time (obtained by subtraction as described above). The peak intensities for both **1** and **4b** subsequently decrease with time, the former much more than the latter (Tacka et al., 2004). Measurements were made with 0.5, 1, 2, 3, 4, and 5 × 10^6 Jurkat cells in 920 μl, usually for 20 h. Peak intensities for **4b** were converted to concentrations of the monocarbonato complex, and [**4**] was fit to an exponential function of time, allowing estimation of the concentration of **4** remaining after the cells rapidly modified a portion of the compound (i.e., at t = 0).

The peak intensity for species **1** alone is calculated as half the difference between the intensity of the main peak (**1** +

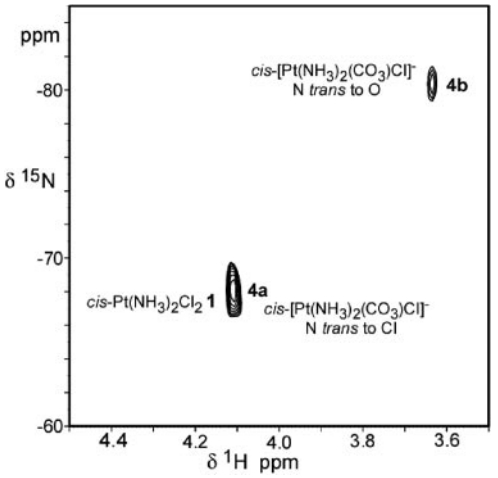


Fig. 1. ^{1}H-^{15}N HSQC NMR spectrum at 37°C of ^{15}N-labeled cisplatin, *cis*-[Pt(NH$_3$)$_2$Cl$_2$] (**1**), in culture media. Also present is the monocarbonato compound *cis*-[Pt(NH$_3$)$_2$(CO$_3$)Cl]$^-$ (**4**). The peak for nitrogen *trans* to oxygen is **4b**, and the peak for nitrogen *trans* to chloride, which is under the peak for **1**, is **4a**. The total platinum concentration is 65 μM.

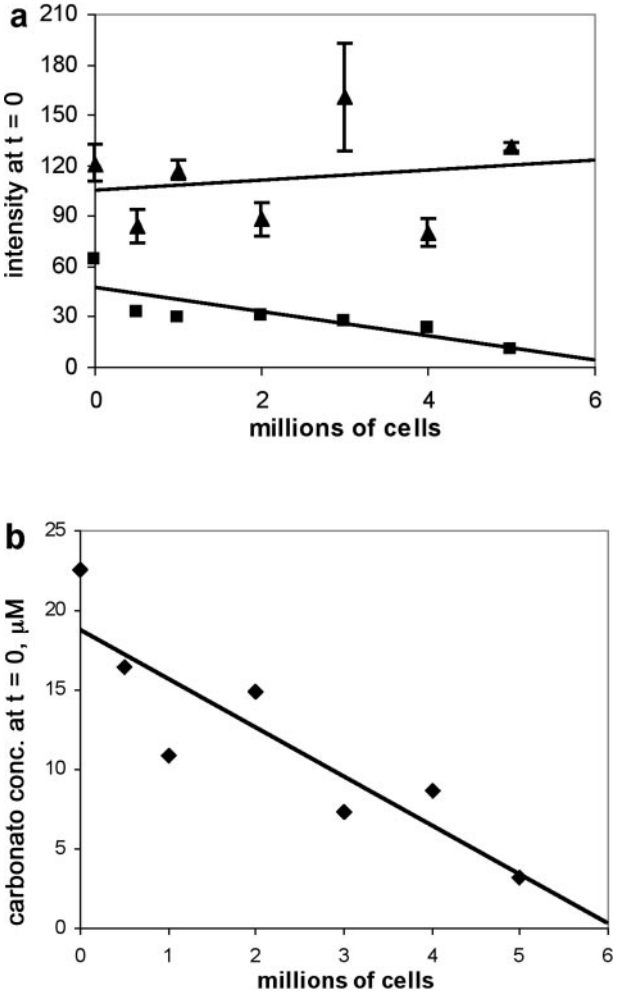


Fig. 2. a, apparent HSQC NMR peak intensities in arbitrary units for the dichloro species *cis*-[Pt(NH$_3$)$_2$Cl$_2$] (**1**) (triangles) and the monocarbonato complex *cis*-[Pt(NH$_3$)$_2$(CO$_3$)Cl]$^-$ (**4**) (squares) at t = 0, after the addition of various numbers of Jurkat cells, obtained by extrapolation from measurements out to t = 10 h (see *Materials and Methods*). Total platinum concentration is 65 μM. Error bars are calculated from exponential fits of concentration versus time. The slopes of the best-fit lines are essentially zero for **1** and definitely negative for **4**. b, concentration of **4** in culture media containing 65 μM total platinum immediately after the addition of Jurkat cells, obtained by extrapolating the HSQC NMR peak intensity for **4b** in the presence of cells to t = 0. Concentration of **4** is plotted versus the number of Jurkat cells, with a least-square linear fit shown. The concentration of **4** apparently becomes 0 at ~6.1 × 10^6 cells.

4a) and the intensity of the **4b** peak. In Fig. 2a, the resulting peak intensities, extrapolated to $t = 0$, are plotted versus the numbers of cells present, with linear fits. The error bars correspond to statistical errors from the fits of peak intensities versus time. (Note that with 5×10^6 cells present, the signal from **4b** is not detected, showing that the cells reduce the concentration of **4b** to below the detection limit of the NMR experiment. Because the minimum detectable intensity is slightly below 20 arbitrary intensity units, we have put the intensity of **4b** for 5×10^6 cells as 10 units.) The slope of the linear fit for **1** is zero within statistical error (2.9 ± 6.9), but the slope of the linear fit for **4b** is definitely negative (-7.2 ± 2.3). The zero slope for cisplatin implies that the concentration of **1** at $t = 0$ is independent of the number of cells. Then, noting that, in the absence of cells, the total peak intensity at $t = 0$ corresponds to 65 μM, we can calculate the initial concentration of **1** as 42.5 μM.

Knowing that [**1**] = 42.5 μM at $t = 0$, we can convert intensities to concentrations in each of the sets of HSQC NMR measurements. The apparent concentration of the monocarbonato complex, **4** (i.e., the concentration remaining after rapid modification by the cells) is plotted versus the number of Jurkat cells present (from 0 to 5×10^6) in Fig. 2b. The best-fit line ($R^2 = 0.79$) corresponds to an x-intercept of $6.1 \pm 1.6 \times 10^6$ cells and [**4**] = 18.8 ± 2.0 μM at $t = 0$. Because the volume of the NMR solution is 920 μl, 6.1×10^6 cells can rapidly modify 0.0173 μmol of **4**; i.e., one cell can modify 0.0028 pmol of **4** so it is undetectable in the NMR experiment. As is evident from the results below, modified **4** remains in the culture medium, but it is incapable of being taken up by the cells.

From the signal intensity for **4b** as a function of time after the cells have rapidly modified a portion of the compound, one finds that the concentration of the monocarbonato species decreases slowly (-1.1 ± 0.4 μM h^{-1}). The rate is independent of how many cells are present (a plot of the rate of decrease versus number of cells has a slope of 0.03 ± 0.16 μM h^{-1} per 10^6 cells). Thus, after rapidly modifying a portion of **4** so that it is not detectable in the NMR experiment, the cells seem to have no further effect on the monocarbonato complex. Compound **1** is observed in the presence and absence of cells, and its rate of decrease is independent of the number of cells in the medium, showing that the cells have little or no effect on this species.

That peaks for NH_4^+ and platinum species having nitrogen *trans* to sulfur in the ^{1}H-^{15}N HSQC NMR spectrum are not observed immediately following the disappearance of **4** in culture media strongly suggests that nucleophilic attack by sulfur is not involved in the rapid modification of the monocarbonato complex.

Uptake of Platinum by Cells. In an attempt to determine the fate of **4** after its rapid modification by cells, we exposed 5×10^6 Jurkat cells to cisplatin and measured the amounts of platinum strongly bound to and/or taken up by the cells (not removable by washing), loosely bound to the cells (recovered in the cell wash), and remaining in the culture medium. The results of two series of such measurements, for 65 and 25 μM total platinum, are shown in Fig. 3. Squares correspond to platinum in the medium, Xs to platinum taken up by the cells, and triangles to platinum in the washes. Measured amounts of platinum have been converted to platinum concentrations in the original solution. Thus, because 65 μM total platinum was used in the first experiments, a concentration of 6.5 μM corresponds to 10% of the total amount of platinum added to the system. The concentrations were also renormalized, so the sum of concentrations for each time is 65 μM (Fig. 3a) or 25 μM (Fig. 3b). The lines in Fig. 3 are linear fits to concentration versus time.

In Fig. 3a, the slope of the line for platinum in the wash versus time is essentially zero (-0.012 ± 0.012 μM h^{-1}), the effective concentration being constant at 0.69 ± 0.07 μM. The slope of the line ($R^2 = 0.93$) for platinum strongly bound to cells is 0.55 ± 0.07 μM h^{-1}. Thus, after 1 h, 1.24 μM, which is less than 2% of the total concentration of platinum, was removed from the culture medium by binding, strongly or weakly, to cells. According to the NMR results of Fig. 2, 5×10^6 cells can reduce the concentration of **4** from 18.8 to 3.4 μM in less than ~0.6 h (total platinum concentration, 65 μM). The decrease, 15.4 μM, is much more than 1.24 μM, showing that most of the platinum that becomes undetectable by NMR remains in the culture medium. Analysis of Fig. 3b, for 25 μM total platinum, leads to the same conclusion.

The slope of the line in Fig. 3b for platinum in the wash is essentially zero (-0.010 ± 0.004 μM h^{-1}). The average value of platinum concentration in the wash is 0.13 ± 0.03 μM, compared with 0.69 ± 0.07 μM for the experiment in Fig. 3a. The ratio, $0.13/0.69 = 0.19 \pm 0.06$, is only half the ratio of the total platinum concentrations in the culture medium for the

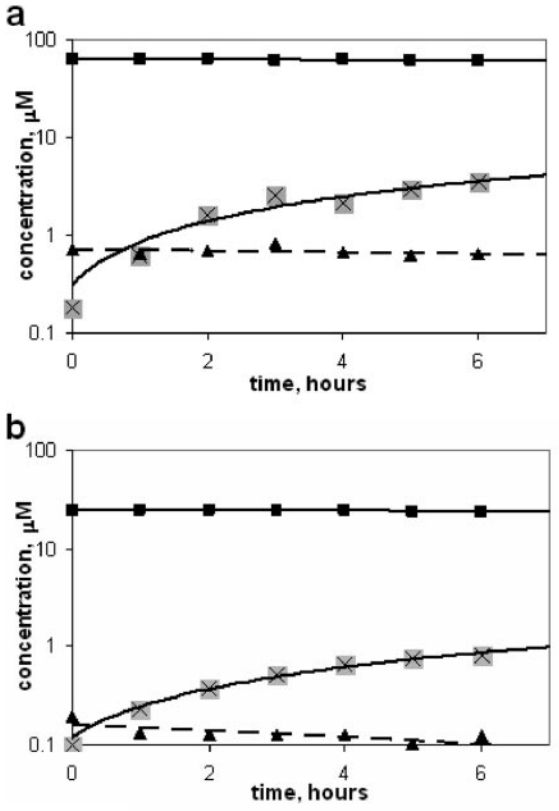


Fig. 3. Five million Jurkat cells were exposed to cisplatin, 65 μM total platinum (a) or 25 μM total platinum (b), for the indicated times. Samples of washed/digested cells, of the wash liquid, and of the culture medium were analyzed for platinum content using ICP-MS, and the results were converted to platinum concentration relative to 65 or 25 μM total platinum. Concentration of platinum strongly bound to or taken up by the cells (X), in the wash (triangles), and in the culture medium (squares) is shown, together with linear least-squares fits to the data.

two experiments, 25 μM/65 μM = 0.38. The slope of the line for platinum strongly bound to cells after exposure to 25 μM total platinum is 0.126 ± 0.007 μM h^{-1}, compared with 0.55 μM h^{-1} for cells exposed to 65 μM total platinum. Both plots are quite linear (R^2 = 0.93 and 0.99 for 65 and 25 μM), and the y-intercepts are close to zero (0.31 ± 0.24 and 0.12 ± 0.03). Thus, there is no sign of saturation with time or concentration.

The ratio of slopes for platinum strongly bound to cells, 0.126/0.55 = 0.23 ± 0.04, is, like the ratio of slopes for the weakly bound platinum (in the wash), much less than the ratio of total platinum concentrations, 0.38. However, if one considers that the concentration of **4** that has been rapidly modified by the cells (15.4 μM) is incapable of binding, strongly or weakly, to cells, or of being taken up by them, one should not use total platinum concentrations, but rather concentrations of unmodified platinum in the culture medium, $[Pt]_o$, the total concentration of platinum remaining in the medium after the cell has modified some of **4**. The ratio of $[Pt]_o$ for the two different total platinum concentrations is (25−15.4 μM)/(65−15.4 μM) = 0.19, which agrees with the observed ratios of slopes (see above) within experimental error. Thus, the modification of the monocarbonato complex **4** by the cells removes it from the pool of platinum that is capable of interacting with cells.

A second series of experiments were performed for both 65 and 25 μM total platinum, with the results shown in Fig. 4. Figure 4A shows the concentrations of platinum in the culture medium as functions of incubation time for the 65 and 25 μM experiments (circles and diamonds, respectively). Best-fit lines are shown; their slopes are close to zero, showing that there is only a small decrease in solution concentration of platinum with time. Figure 4B shows the concentrations of platinum found in the wash solutions as functions of incubation time. The best-fit lines have slopes of 0.046 ± 0.016 and −0.004 ± 0.003 for 65 and 25 μM platinum (that the former is different from zero can be attributed to the large scatter in the points). The average concentrations are, respectively, 0.828 and 0.181, giving a ratio of 0.22, in agreement with 0.19, the ratio calculated for $[Pt]_o$ in the two experiments.

In Figure 4C, we show the concentration of platinum strongly bound to the cells, which remains after washing the cells once with phosphate-buffered saline. For both 25 and 65 μM, the concentration of strongly bound platinum versus time t is highly linear, as in the results of Fig. 3. The linear fits, shown in the graph, are 0.056 + 0.821t (R^2 = 0.995) for 65 μM and 0.135 + 0.195t (R^2 = 0.992) for 25 μM. The ratio of slopes is 0.24, in agreement with the ratio expected if modified **4** cannot interact with cells. Because the intercepts are close to zero (0.135 ± 0.028 and 0.056 ± 0.097), uptake is closely proportional to the time of exposure to the drug. As in the experiments of Fig. 3, there is no sign of saturation for strong binding.

The experimental results shown in Figs. 3 and 4 allow calculation of the rate at which platinum strongly binds to and/or is taken up by Jurkat cells. For 5 × 10^6 cells in 65 μM total platinum, or $[Pt]_o$ = 51.2 μM, we found slopes of 0.55 and 0.82 μM platinum h^{-1} (average, 0.685 μM). Because the volume of the culture medium was 920 μl, 6.3 × 10^{-10} mol of platinum was taken up per hour by 5 × 10^6 cells, corresponding to the binding of 0.000125 pmol of platinum h^{-1} cell^{-1}. For 5 × 10^6 cells in 25 μM total platinum, $[Pt]_o$ = 11.2 μM, we found slopes of 0.13 and 0.195 μM platinum h^{-1} (average, 0.16 μM). This yields a rate of 0.000029 pmol h^{-1} for platinum binding in each cell. The observed ratio of rates of platinum binding is 0.23, which is close to the value of 0.19 calculated from the values of $[Pt]_o$ for the two concentrations.

Likewise, we found that, when 65 μM cisplatin is used, 0.828 μM cisplatin is loosely attached to cells but removable by washing, independent of the incubation time; when 25 μM cisplatin is used, 0.181 μM cisplatin is loosely attached but removable by washing. Because the ratio is 0.22, which agrees with the ratio calculated from the values of $[Pt]_o$, the loosely bound platinum is on the exterior surface of the cell. Platinum must reach the cell surface before it can enter, which supports the idea that modified **4** is harmless to cells. Thus, the chemical modification of **4** is an important component of how the cell resists exposure to cisplatin.

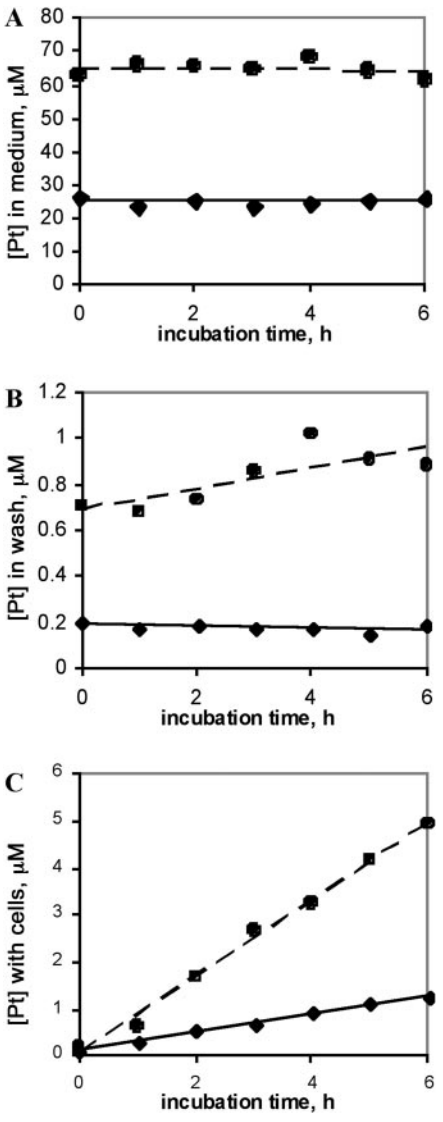


Fig. 4. Five million Jurkat cells were exposed to 65 or 25 μM total platinum for the indicated times. Samples of washed/digested cells, of the wash liquid, and of the culture medium were analyzed for platinum content using ICP-MS, and the results were converted to platinum concentration relative to 65 μM (circles) or 25 μM (diamonds) total platinum. A, concentration of platinum in the medium for the two experiments, with linear fits. B, concentration of platinum found in wash liquid, with linear fits. C, concentration of platinum taken up by cells, with linear fits.

As noted, the amount of loosely bound platinum is roughly independent of incubation time but proportional to $[Pt]_o$. This suggests strongly that a steady state is established, in which the net rate at which platinum reaches the cell surface from bulk solution is equal to the rate at which surface-bound platinum enters the cell. Because the former rate is proportional to $[Pt]_o$ and the latter rate is proportional to the concentration of surface-bound platinum, the concentration of surface-bound platinum is proportional to $[Pt]_o$ as we have found. In addition, because $[Pt]_o$ does not change much, the concentration of surface-bound platinum remains constant

Platinum Binding to DNA. In connection with a study (Tacka et al., 2004) of exposing Jurkat cells to different area-under-curve dosages, we previously measured the rate of formation of platinum-DNA adducts in Jurkat cells exposed to cisplatin. Samples of 10^7 cells were incubated with various concentrations of cisplatin at 37°C for 1, 1.5, or 3 h, after which the drug was removed and the DNA was immediately extracted from the cells, and the number of platinum adducts was measured. Because the area-under-curve is the product of the cisplatin concentration and the exposure time, cisplatin concentrations of 0, 5, 10, 15, 25, 30, 45, 50, and 75 μM were used.

The results are shown in Fig. 5, where the number of platinum adducts on 10^6 nucleotides is plotted versus the total platinum concentration in the culture medium, with least-squares linear fits. Triangles and gray solid line are for 1-h incubation, circles and dashed line are for 1.5-h incubation, and diamonds and solid line are for 3-h incubation. Each point is the average of two or three measurements, the error bars being the SD in each case. For each incubation time, the number of adducts per 10^6 base pairs, N_a, is proportional to total cisplatin concentration, C, in micromolar. For 1-h exposure, $N_a = 0.46C + 3.30$ ($R^2 = 0.961$); for 1.5-h exposure, $N_a = 0.71C + 2.60$ ($R^2 = 0.968$); for 3-h exposure, $N_a = 1.70C + 2.22$ ($R^2 = 0.981$). Furthermore, the number of platinum-DNA adducts is proportional to incubation time: if the three slopes [in adducts on 10^6 nt for 1 μM cisplatin] are plotted versus incubation time, they fall on a straight line ($R^2 = 0.997$) with slope equal to 0.628 ± 0.032 and y-intercept equal to -0.194 ± 0.064.

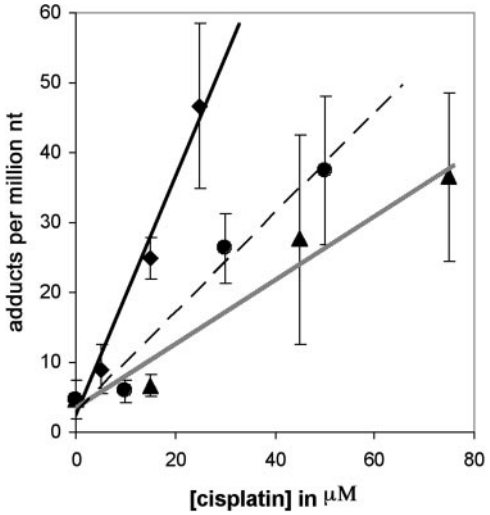


Fig. 5. Number of platinum-DNA adducts per 10^6 nucleotides for Jurkat cells exposed to the indicated concentrations of cisplatin (0, 5, 10, 15, 25, 30, 45, 50, and 75 μM, total concentration of platinum) for 1, 1.5, or 3 h. Lines are least-squares linear fits. Triangles and gray solid line, 1-h incubation; circles and dashed line, 1.5-h incubation; and diamonds and solid line, 3-h incubation with cisplatin. The total concentration of cisplatin in the culture medium is given. Average and SD of two or three measurements are shown.

Thus, the rate of formation of DNA adducts is 0.628 adducts h^{-1} for 10^6 nt for 1 μM total cisplatin. Assuming 6×10^9 nt in a cell (two single strands, each, 3×10^9 nt), this means a 1-h exposure to 65 μM total platinum would yield 2.45×10^5 platinum-DNA adducts per cell. As described above, we calculated the rate at which platinum actually enters, or binds tightly to, one cell h^{-1} as 0.000125 pmol of platinum when exposed to 65 μM cisplatin. According to this rate, a 1-h exposure would result in 75×10^6 platinum atoms entering the cell, 300 times the number of platinum-DNA adducts formed. Thus, only ~0.33% of the platinum that enters the cell is bound to genomic DNA. Thus, most of the intracellular platinum is bound to proteins or small molecules [e.g., glutathione (Eastman, 1999)] or it remains inside the cell in a form that is not readily removed by washing.

Discussion

From our study, it is not possible to tell whether cisplatin (**1**), the monocarbonato complex (**4**), or both are entering the cell. However, DNA binding most likely takes place through **4**, or possibly its protonated bicarbonate analog. For **1** to bind to DNA, it would first have to aquate to form **2,** which, as shown in Scheme 1, is in a rapid proton equilibrium with **3** (Miller and House, 1990; Berners-Price and Appleton, 2000). Because dissolved carbon dioxide in the cytosol could rapidly react with **3** to produce the monocarbonato complex **4** (Palmer and van Eldik, 1983; Acharya et al., 2004; Centerwall et al., 2005), **1** on entering the cell would be rapidly transformed into **4**, which would interact with cellular components, including DNA. We recently showed that cisplatin reacts with pBR233 DNA in carbonate buffer to produce monofunctional DNA adducts that do not induce the same conformational changes in DNA as the well known bifunctional 1, 2 intrastrand cross-link formed in the absence of carbonate (Binter et al., 2006). It is widely accepted that the nature of the platinum lesions on DNA is important for the induction of apoptosis (Eastman, 1999; Wang and Lippard, 2005), so this observation is significant and suggests additional study.

Because more than 99% of the platinum that enters cells does not bind to DNA, it must react with other substances in the cytosol or bind to extranuclear cell structures. Some authors (Lindauer and Holler, 1996) have distinguished between "chemically reactive platinum", defined as intracellular platinum able to react with calf thymus DNA, and "inactive platinum". The latter may be inactive because it has reacted with substances in the cytosol; it is possible that the reacted intracellular platinum is responsible for the termination of the cellular defense mechanism, which modifies **4**. As shown above, after the rapid decrease in the extracellular concentration of **4** caused by cells, [**4**] remains essentially constant for several hours, indicating that the capacity of the cells to produce the agent responsible for modification has been impaired.

It is clear from the concentration dependence of platinum uptake that the modified **4** is harmless to cells because it does not enter them. It is conceivable that, if the concentration of

4 is low enough and the number of cells high enough, all of **4** can be modified by the cells. Because the rate of production of **4** from **1** is slow (Miller and House, 1990; Berners-Price and Appleton, 2000), [**4**] will remain close to zero for several hours. If, as we have argued above, **4** is the species that enters cells, no platination of DNA could occur for several hours. This may be supported by the data of Fig. 5, which shows the number of platinum-DNA adducts resulting from incubating 10^7 cells with various concentrations of cisplatin for various times. The number of adducts from a 1.5-h incubation with 10 μM cisplatin, or from a 1-h incubation with 15 μM cisplatin, is essentially the same as for incubation with no cisplatin. However, the experimental errors are too large to conclude definitively that no platinum-DNA adducts are formed.

Comparing uptake rates of platinum is complicated by the many different ways of defining and measuring uptake. If uptake is defined as the difference between the concentrations of platinum in the medium in the presence and absence of cells, it is subject to the objection that the cells may modify the form of platinum, making it undetectable by the measurement technique used. A better definition is the amount of platinum actually found associated with cells, determined by analyzing the cells for platinum after removal of the culture medium. As has been pointed out (Ghezzi et al., 2004), this combines platinum that has actually entered the cells and platinum that is attached to the cell surfaces. One can partly distinguish between the two by washing the cells and analyzing the wash liquids for platinum, as we have done. The platinum that has actually entered the cells may be further divided into platinum bound to the nuclear DNA, platinum in an "active" form in the cytosol (Lindauer and Holler, 1996), and so on. In addition, platinum uptake has been reported as intracellular concentration of platinum, as platinum atoms per mass of cellular protein, or otherwise.

A review of earlier work on intracellular accumulation of cisplatin was given by Gately and Howell (1993). Mann et al. (1990) measured accumulation or uptake of platinum in sensitive human ovarian carcinoma cells and in a cisplatin-resistant variant, when exposed to 500 μM cisplatin. The cells exhibited very high accumulation rates during the first minute of exposure, but rates then dropped sharply, to 51 $\pm$ 22 and 34 $\pm$ 10 pmol of platinum min^{-1} for each milligram of protein for sensitive and resistant cells. Assuming that a cell is ~40% protein (Stocker and Dietrich, 1996) and has a mass of 2 ng, 51 pmol of platinum per mg of protein min^{-1} equals 0.0024 pmol of platinum $cell^{-1}$ h^{-1}. Because uptake rates were found to be proportional to drug concentration, the uptake rates for sensitive and for resistant cells in 65 μM cisplatin would be 3.2×10^{-4} and 2.1×10^{-4} pmol of platinum $cell^{-1}$ h^{-1}, slightly higher than our result of 1.2×10^{-4} pmol of platinum $cell^{-1}$ h^{-1}.

Jennerwein and Andrews (1994) examined uptake of platinum by 2008 human ovarian carcinoma cells exposed to the dichloro or the aquated forms of cisplatin. After 1-h exposure to 100 μM drug, intracellular platinum accumulation was approximately the same for all forms, averaging 13.4 nmol for each milligram of protein. This corresponds to an uptake rate of 1.07×10^{-5} nmol platinum $cell^{-1}$ h^{-1} for 100 μM drug or 6.7×10^{-3} pmol of platinum $cell^{-1}$ h^{-1} for 65 μM drug, much higher than found by others. These authors found a substantial difference in the amount of platinum bound to DNA after exposure of the cells to 50 μM unaquated and aquated cisplatin for 1 h. The levels were 27.8 $\pm$ 3.0 pg of platinum per μg of DNA for exposure in regular medium and 53.2 $\pm$ 4.8 pg of platinum per μg of DNA for exposure to preaquated cisplatin in chloride-deficient medium. Because cisplatin forms carbonato complexes via the aquo species (Centerwall et al., 2005), uptake and DNA binding of cisplatin-carbonato compounds were probably measured in these studies. Assuming 6×10^9 nt $cell^{-1}$ and ~300 g mol^{-1} nt^{-1}, each cell has 3.0 pg of DNA. Thus, the above-mentioned rates correspond to 1.08×10^{-4} and 2.07×10^{-4} pg of platinum bound to DNA $cell^{-1}$ h^{-1} when exposed to 65 μM cisplatin. Our rate is somewhat smaller, 7.7×10^{-5} pg of platinum bound to DNA $cell^{-1}$ $h^{-}]^1$.

Johnson et al. (1996) studied the rates of cisplatin accumulation and platinum-DNA adduct formation in five human hepatoma cell lines exposed for 4 h to cisplatin concentrations between 0 and 200 μM. Platinum accumulation was found to be proportional to cisplatin concentration up to 200 μM. For the parent cell line, platinum accumulation was 1.2 μg per 10^6 cells for a 4-h exposure to 200 μM cisplatin and 5 to 14 times lower for the resistant strains. This corresponds to 0.0005 pmol $cell^{-1}$ h^{-1} for 65 μM, approximately 4 times our 1.2×10^{-4} pmol $cell^{-1}$ h^{-1}. The amount of platinum-DNA adducts formed was also proportional to cisplatin concentration. In the parent cell line, 560 pg of platinum was found per microgram of DNA after a 4-h exposure to 200 μM cisplatin; in a resistant line, the platinum content was 7-fold lower. Assuming 3.0 pg of DNA $cell^{-1}$, the rate of adduct formation at 65 μM cisplatin becomes 7×10^{-7} pmol of platinum $cell^{-1}$ h^{-1}. We measured 4×10^{-7} pmol of platinum $cell^{-1}$ h^{-1}.

Trying to determine whether uptake of platinum drugs was passive or active, Pereira-Maia and Garnier-Suillerot (2003) studied the uptake of cisplatin and its aquated forms by sensitive and resistant small lung cancer cells. For 100 μM cisplatin, the uptake rate was 6.2×10^{-20} mol $cell^{-1}$ s^{-1} for sensitive cells and 3.7×10^{-20} mol $cell^{-1}$ s^{-1} for resistant cells. The former rate corresponds to 1.45×10^{-4} pmol of platinum $cell^{-1}$ h^{-1} at 65 μM cisplatin, the latter to 0.87 $\times$ 10^{-4} pmol of platinum $cell^{-1}$ h^{-1}, bracketing our value.$\times$

As we show here, Jurkat cells in culture rapidly modify **4** in the culture medium, making the compound undetectable by HSQC NMR and incapable of binding to the cell. This process seems to be a hitherto undocumented defense by the cell against a toxic form of cisplatin, and it could be an additional mechanism of resistance to cisplatin. However, unlike other resistance mechanisms that operate after the drug enters the cell [e.g., reaction with thiols or enhanced platinum adduct repair (Kartalou and Essigmann, 2001; Siddik, 2003; Brabec and Kasparkova, 2005)], this mechanism of detoxification seems to take place outside the cell and to be selective for one form of the drug, **4**, in the culture medium. It is also possible that, instead of being modified outside the cell, **4** is absorbed by the cell, modified inside, and ejected into the medium on a rapid time scale. Additional work will be needed to determine the mechanism by which **4** is modified, the nature of the products formed, and to what extent the process is related to a previously identified resistance mechanism broadly described (Kartalou and Essigmann, 2001) as "drug efflux" from the cell.

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