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Modification of carboplatin by Jurkat cells

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Abstract

Using [^{1}H,^{15}N] heteronuclear single quantum coherance (HSQC) NMR and ^{15}N-labeled carboplatin, **1**, we show that Jurkat cells affect the rate of disappearance of the HSQC NMR peak in culture medium for this Pt^{2+} anticancer drug. The decay or disappearance rate constant for **1** in culture medium containing cells is $k_1 = k_c[CO_3^{2-}] + k_m + k_u N$, where k_c is the rate constant for reaction of **1** with carbonate in the medium, k_m is the rate constant for reaction of **1** with all other components of the medium, and k_u is the rate constant for reaction of **1** with cells having a number density N in the medium. Since Jurkat cells only take up a small amount of the platinum present in the medium (<1%), the observed disappearance of the HSQC NMR peak for **1** cannot be due to uptake of carboplatin by the cells.
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1. Introduction

Carboplatin, [Pt(NH$_3$)$_2$(CBDCA-O,O')], **1**, where CBDCA is cyclobutane-1,1-dicarboxylate, Fig. 1, is a Pt^{2+} anticancer drug in wide clinical use [1]. Using 2D [^{1}H,^{15}N] heteronuclear single quantum coherence (HSQC) NMR, 1D ^{13}C and ^{1}H NMR, and UV–visible spectroscopy, we showed that carboplatin reacts with carbonate ion, present in culture media, blood and the cytosol, to produce carbonato carboplatin, *cis*-[Pt(NH$_3$)$_2$(O-CBDCA)-(CO$_3$)]$^{-2}$, and other carbonato complexes [2,3]. *In vitro* cytotoxicity studies showed that the carbonato complexes are more toxic to human neuroblastoma (SK-N-SH), renal proximal tubule (HK-2) and Namalwa-luc Burkitt's lymphoma cells than is intact **1**, suggesting that small amounts of carbonato complexes could form in therapy and be the species responsible for the antitumor effects of carboplatin

[3]. Earlier we also showed that carbonate reacts with the related drug, cisplatin, and that one of the complexes is selectively modified by Jurkat cells, immortalized T lymphocytes, using an extra-cellular mechanism [4,5].

We here use [^{1}H,^{15}N] HSQC NMR and ^{15}N-labeled **1** to show that the rate of disappearance of carboplatin in the medium is affected not only by substances present in the culture medium but also by an unknown substance(s) released by the cells to the medium. As with previously reported experiments with cisplatin [4], no HSQC NMR active product peaks are observed in the NMR experiment with carboplatin and Jurkat cells. Since Jurkat cells take up only a small fraction of the total amount of platinum present in the medium [3], the disappearance of **1** cannot be due to simple absorption of the compound by the cells. We also demonstrate that the results of the cell/NMR experiments are independent of the nature of the atmosphere above the cells. Rate constants for the disappearance of **1** in the presence of Jurkat cells in a static atmosphere (closed NMR tube) fit well ($R^2 = 0.96$) with the previously reported rate constant for the disappearance of carboplatin
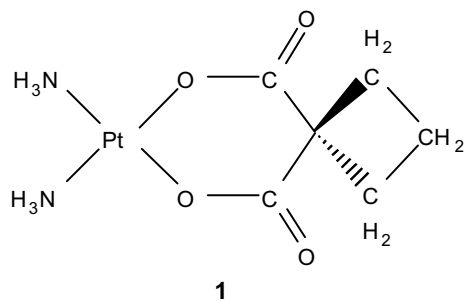

Fig. 1. Structure of carboplatin.

in the presence of 4.5×10^7 cells mL^{-1} kept under standard conditions in a humidified, 5% CO_2 atmosphere in a incubator [2].

2. Materials and methods

K$_2$PtCl$_4$, H$_2$CBDCA, ^{15}NH$_4$Cl and D$_2$O (99.9%) were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) and RPMI-1640 (15–040) were purchased from Mediatech (Herndon, VA). ^{15}N-labeled carboplatin was prepared as previously described [2]. [^{1}H,^{15}N] HSQC NMR spectra were recorded on a Bruker Avance 500 MHz NMR equipped with a 5 mm triple axis probe in the earlier described manner [5]. Peak volumes in arbitrary units, excluding ^{195}Pt satellites, were calculated using Bruker software. Each kinetic run consisted of ten successive NMR measurements (each 60 min in length, ns = 48) for a total duration of ∼10 h. The ^{15}N chemical shift of **1** was referenced externally to 1 M (^{15}NH$_4$)$_2$SO$_4$ in 5% D$_2$O which was acidified to pH ∼1 by the addition of H$_2$SO$_4$. The ^{1}H chemical shift of **1** was referenced externally to 3-trimethylsilyl propionic acid-d$_4$ sodium salt (TSP) in a pH 7.15, 23 mM sodium carbonate buffer. Freshly prepared labeled carboplatin in water was added to culture medium (RPMI + 10% FBS, 100 µg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM S-glutamine) containing either 2.7, 8.2, 21.2 or 27.2×10^7 Jurkat cells per mL, in a final volume of 920 µl, and spectra were recorded in 5% D$_2$O at 37 °C. Jurkat cells used were ∼97% viable, measured by light microscopy, using a hemacytometer under standard trypan blue conditions [6].

3. Results and discussion

As is shown in Fig. 2, ^{15}N-labeled carboplatin exhibits a single HSQC NMR peak due to the two symmetry-equivalent ammonia molecules. The pseudo-first order decay constant, k_1, for the disappearance of carboplatin in culture medium containing Jurkat cells was determined by fitting a plot of carboplatin concentration (calculated from the HSQC NMR peak intensity) versus time to a line and dividing the slope of the linear fit by its y-intercept. The values obtained for k_1 are given in Table 1, with statistical errors from the linear fits, and are plotted versus cell den-
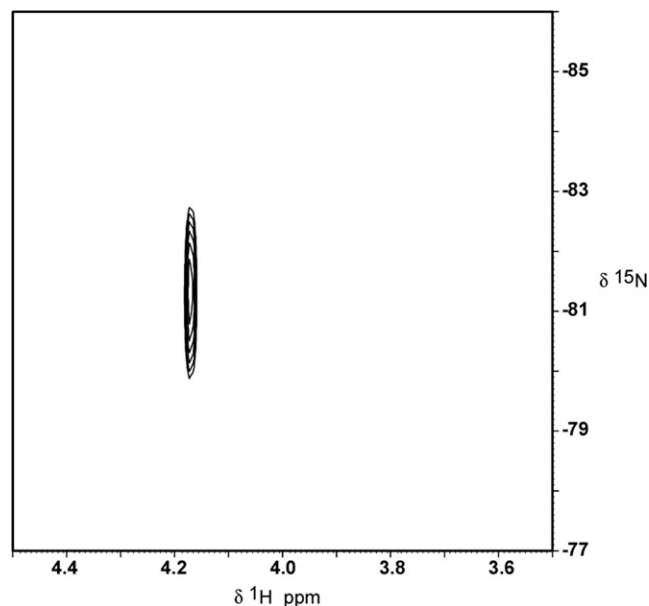

Fig. 2. [^{1}H,^{15}N] HSQC NMR spectrum of 110 µM ^{15}N-carboplatin in culture medium (RPMI + 10% FBS, 100 µg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM S-glutamine) containing 2.7×10^7 Jurkat cells mL^{-1}, pH = 7.4, 9.6 h after the addition of carboplatin to the medium containing the cells.

Table 1
Pseudo-first order decay constant, k_1, for the disappearance of carboplatin in culture medium containing Jurkat cells determined using [^{1}H,^{15}N] HSQC NMR

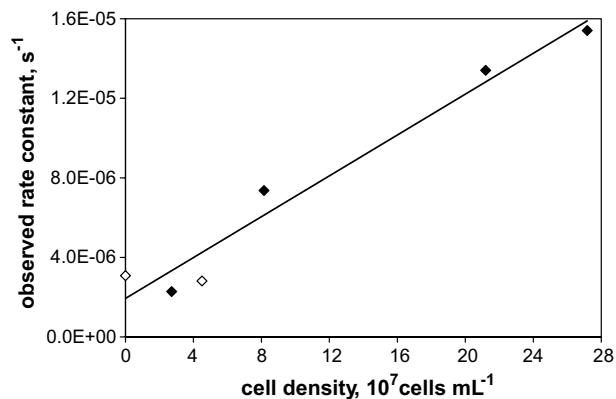| Jurkat cell density (cells mL^{-1})	k_1 (s^{-1})
2.7×10^7	$2.28 \pm 0.60 \times 10^{-6}$
8.2×10^7	$7.36 \pm 1.02 \times 10^{-6}$
21.2×10^7	$13.41 \pm 2.94 \times 10^{-6}$
27.2×10^7	$15.41 \pm 1.67 \times 10^{-6}$

Fig. 3. Observed rate constant of the disappearance of carboplatin obtained from HSQC NMR as a function of number of cells per mL. Open diamonds are k_1 values already reported by us [2]. The best fit line, $R^2 = 0.96$, is $k_1 = (1.94 \times 10^{-6} + 5.14 \times 10^{-14} \text{ mL cell}^{-1}N)$ s^{-1}, so that $k_u = 5.14 \times 10^{-14}$ mL cell^{-1} s^{-1}.

sity in Fig. 3. Also plotted in Fig. 3 are the previously reported k_1 values for the disappearance of carboplatin in

culture medium (no cells) and in culture medium containing 4.5×10^7 cells mL^{-1} kept under standard conditions in a humidified 5% CO_2 atmosphere in an incubator [2]. In the previously described cell experiment [2], aliquots of cells plus medium were removed from the culture flask, cells were separated from the medium by centrifugation, 5% D_2O was added to the medium and NMR data was collected for 0.5 h. As can be seen in Fig. 3, all the data points are consistent with the best fit ($R^2 = 0.96$) line $k_1s = 1.94 \times 10^{-6} + 5.14 \times 10^{-14}$ (mL cell^{-1})N, demonstrating that cell studies will yield the same results regardless if the reaction with drug is done in a 5 mm capped NMR tube or in a humidified, 5% CO_2 incubator.

A striking feature of the reaction of **1** in these studies is that no new HSQC NMR peaks associated with products are observed. As we, as well as Sadler and coworkers, have shown [2,7–9], reaction of **1** with nucleophiles lifts the equivalency of the two ammonia molecules to produce new peaks, the positions of which are sensitive to the nature of the ligands *trans* to the ammonia molecules. Sulfur in the form of a thiol (cysteine) or thioether (methionine) is the most potent nucleophile for Pt^{2+} present in culture media. While a sulfur ligand *trans* to an ammonia molecule would make the latter vulnerable to a substitution reaction due to the *trans effect*, these complexes, with N *trans* to S, are sufficiently stable to be easily detected at ~ -40 ppm (N) using HSQC NMR [4,7–11]. No products of this type or of any other species were observed in the reaction.

The prospect that the disappearance of the HSQC NMR peak for **1** in the presence of cells is due to absorption of carboplatin by the cells is highly unlikely. As we earlier showed, Jurkat cells under conditions similar to those used here remove platinum species from solution at a rate which is only $16.6 \pm 4.2 \times 10^{-18}$ mol Pt h^{-1} cell^{-1} [3]. This rate of platinum removal by cells is too slow to show an observable effect on the total pool of **1** in the medium above the cells during the time course of these experiments.

In the presence of culture medium without cells, the concentration of carboplatin [1] should obey

$$\frac{d[\mathbf{1}]}{dt} = -k_c[1][CO_3^{2-}] - k_m[1]$$

where k_c is the rate constant for reaction of **1** with carbonate in the medium, a second order process, which behaves like a pseudo-first order reaction because $[CO_3^{2-}]$ is constant under the reaction conditions used [2]. The quantity k_m is the rate constant for reaction of **1** with other substances in the medium, which is assumed to be a first order process. For example, earlier studies showed that FBS, a suite of proteins needed for cell growth, is also a significant factor causing the disappearance of **1** in culture media [2].

If cells, or some substance released by them, can modify carboplatin so that it does not give an NMR signal, a term $-k_u[\mathbf{1}]N$ should be added to the equation for d[**1**]/dt, where N is the density of cells in the medium. The solution to the resulting equation is an exponential decay,

$$[\mathbf{1}] = [\mathbf{1}]_0 \exp\{-(k_c[CO_3^{2-}] + k_m + k_uN)t\} = [\mathbf{1}]_0 \exp\{-k_1t\}$$

where the effective decay constant $k_1 = k_c[CO_3^{2-}] + k_m + k_uN$. The observed values of k_1, shown in Fig. 3, depend linearly on cell density. The y-intercept, $1.94 \pm 0.76 \times 10^{-6}$ s^{-1} represents $k_c[CO_3^{2-}] + k_m$; from the slope, we obtain $k_u = 5.14 \pm 0.52 \times 10^{-14}$ mL cell^{-1} s^{-1}. When the density of cells is $\geqslant 8.2 \times 10^7$ cells mL^{-1}, the reaction of carboplatin with cells is more important than reaction of drug with carbonate or other substances in the culture medium. Since, as noted, direct removal of **1** by cells is unimportant, the reaction must involve a substance released by the cells.

Earlier studies by us using cisplatin [4,5] showed that Jurkat cells can rapidly modify the mono-carbonato complex, *cis*-[Pt(NH$_3$)$_2$(CO$_3$)Cl]$^-$, converting it into a substance that is not capable of binding to the cells and is undetectable using HSQC NMR. Moreover, the amount of modification of the mono-carbonato complex is dependent on the number of cells and recent studies by our group (Centerwall et al., personal communication) show that the platinum-modifying substance is released to the culture medium above the cells. Unlike the relatively slow decay rate observed for carboplatin in the presence of cells in these experiments, the rate of modification of the cisplatin mono-carbonato complex by Jurkat cells is extremely rapid, occurring within ~ 0.6 h of the addition of drug to the culture medium above the cells [4,5]. It seems likely that both drugs are exposed to the same cell-released modifying substance, but since carboplatin has a bidentate chelate ring which blocks nucleophiles from attacking the axial sites of the compound, it is much less susceptible to chemical modification than is the cisplatin mono-carbonato species. Clearly, additional work is needed to determine the nature of the species released by the cells and products formed in the reaction.

4. Conclusion

Jurkat cells release a substance to the culture medium that reacts with carboplatin, converting it into a substance that, under the conditions of the experiment, is not detectable using HSQC NMR. This disappearance from HSQC NMR is identical to that observed for a mono-carbonato complex of cisplatin except that, due to structural differences between cisplatin and carboplatin, the rate of reaction with carboplatin is much slower than with cisplatin.

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