Platinum Antitumor Agents

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Methionine Can Favor DNA Platination by *trans*-Coordinated Platinum Antitumor Drugs**

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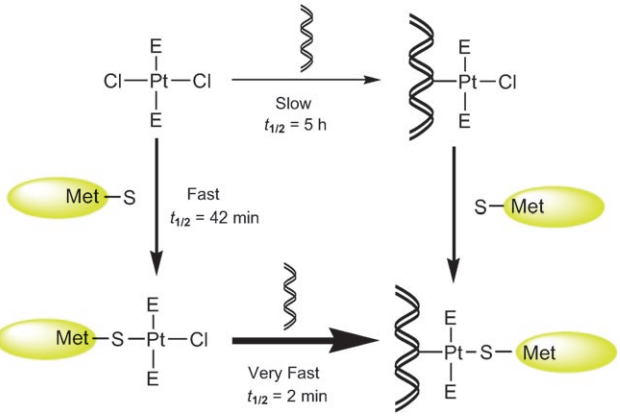
Cisplatin (*cis*-DDP) and its *cis*-coordinated analogues, carboplatin and oxaliplatin, have been successfully used in the treatment of testicular and other solid tumors, but applications are restricted by side effects and intrinsic and acquired resistances.[1–3] The discovery of *trans*-coordinated platinum complexes with antitumor activity provides a novel approach for cancer chemotherapy.[4,5] Among several types of *trans*-platinum complexes, *trans*-[$PtCl_2${*E*-HN=C(OCH_3)CH_3}$_2$] (*trans-EE*) raised particular interest because of its higher cytotoxicity than the *cis* isomer and its activity towards several *cis*-DDP-resistant tumor cells.[4] Mechanistic studies indicated that *trans-EE* has different DNA binding modes relative to *cis*-DDP,[6,7] although their reaction rates were similar.[8] DNA modified by *trans-EE* could not be recognized by high-mobility group (HMG), the protein that interferes with DNA repair of *cis*-DDP adducts, whereas histone H1 could bind to *trans-EE*-modified DNA and prevent DNA polymerization and repair.[9] A recent study also indicated that methionine was the preferable binding site of *trans-EE* in the reaction with cytochrome *c*, and different binding modes were observed between *cis*- and *trans*-platinum complexes.[10]

Many cellular molecules, including proteins, peptides, and also some small molecules, can play significant roles in the functioning of and resistance to drugs, such as DNA platination, drug transport, and efflux.[2,11,12] Sulfur-containing proteins are of special interest because of their high affinity for platinum, their abundance (e.g. albumin),[13,14] and their involvement in metal-ion transport (e.g. the copper transporter protein CTR1, which contains methionine-rich extracellular motifs and appears to be involved in platinum-drug transport through the cell membrane).[11,15] Kinetic studies indicated that the S platination of L-methionine (Met) or *N*-acetyl-L-methionine (AcMet) was kinetically preferred, whereas N7 coordination of guanine was thermodynamically favored.[16] Studies using the model compound $[PtCl(dien)]^+$ showed that the migration of platinum from S-Met to N7-guanine (G–N7) was fairly slow ($t_{1/2}$ = 21–147 h at 310 K depending on DNA sequence), which was obviously slower than the direct DNA platination by $[PtCl(dien)]^+$.[17,18] Although Met could slightly increase the rate of platination of *cis*-DDP to guanosine monophosphate (GMP), the reaction with synthetic DNA showed that the presence of Met actually inhibited platination on both single strand (ss) and double strand (ds) DNA.[19]

Herein we show that the platination rates of both GMP and DNA are substantially enhanced by a Met ligand bound to *trans-EE*. Moreover the reaction is highly pH-dependent. This enhancement has been observed for all nucleotides used in this work, including monomeric GMP, synthetic ss- and ds-DNA, and natural DNA. It has been observed that the formation of a Met intermediate is about seven times faster than G–N7 platination (Figure S1 in the Supporting Information). Accordingly, DNA platination is significantly faster via a Met intermediate (Scheme 1). On the basis of activity studies and the formation of this type of adducts also in the cellular system, it is suggested that the mechanism of *trans-EE* could differ substantially from that of conventional *cis*-platinum compounds.



Scheme 1. Kinetic pathway for the formation of *trans-EE* adducts. *E* = *E*-HN=C(OCH_3)CH_3. $t_{1/2}$ values are based on the AcMet and GMP reactions at pH 7.0.

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The monofunctional complex *trans-EE*/AcMet was prepared by reaction of *trans-EE* with AcMet in a 1:1 ratio. The coordination of AcMet could be directly deduced from the downfield shift of the SCH_3 signal upon platinum binding (shift from $\delta = 2.1$ to 2.4 ppm). The formation of $[PtCl\{E\text{-}HN{=}C(OCH_3)CH_3\}_2(AcMet)]^+$ was also confirmed by ESI-MS spectra (*m*/*z*: calcd 568.108, obsd 568.140; Figure S2 in the Supporting Information). This compound is stable in acidic solution (pH 2.4) at room temperature for over two weeks (monitored by NMR spectroscopy). The reaction between *trans-EE*/AcMet and GMP was monitored by ^{1}H NMR spectroscopy. An approximately 0.5 ppm downfield shift of the G-H8 signal, typical of G–N7 platination, was observed. The ESI-MS spectrum confirmed the formation of the ternary complex GMP/*trans-EE*/AcMet (*m*/*z*: calcd 894.182, obsd 894.300). The kinetic process was monitored by the disappearance of free GMP (Figure 1).

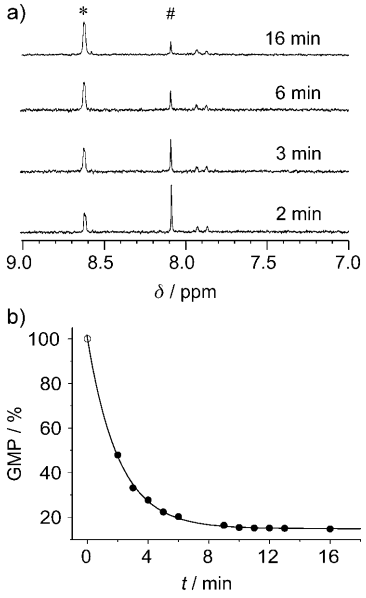

Figure 1. Reaction of GMP with *trans-EE*/AcMet in 20 mM phosphate buffer in D_2O (pH 7.0) monitored by 600 MHz ^{1}H NMR spectroscopy at 298 K. a) 1D ^{1}H NMR spectra of the G–H8 region. Symbols denote the H8 signal from free GMP (#) and platinated GMP (*). The reaction time is given for each spectrum. b) Integration of H8 peaks from free GMP as a function of the reaction time. Data were fitted with a first-order exponential decay and a *k* value of 8.1×10^{-3} s^{-1} ($t_{1/2}\approx2$ min) was obtained.

Surprisingly, G–N7 platination by *trans-EE*/AcMet is extremely fast ($t_{1/2}\approx2$ min). This reaction is over two orders of magnitude faster than the direct reaction between *trans-EE* and GMP observed previously[8] and confirmed in this work ($t_{1/2}\approx5$ h). This result indicates that the presence of a sulfur-containing ligand induces a remarkable enhancement in the rate of G–N7 platination by the *trans*-platinum complex. In contrast, in the case of *cis*-DDP, the rate of GMP platination increases only marginally in the presence of a Met ligand.[20] Thus, the present work demonstrates that S-donor molecules can play significantly different roles in the cellular processing of *trans*-platinum drugs compared with antitumor drugs with *cis* geometry.

To further investigate the role of Met in the reaction between *trans-EE* and nucleotides, the competition reaction between GMP and AcMet was carried out. The results showed that the ternary complex was dominant throughout the reaction, whereas binary complexes containing GMP or AcMet alone were formed only in a small amount (80% ternary adduct, 7% GMP/*trans-EE* adduct, and 13% *trans-EE*/AcMet adduct after reaction for 12 h; Figure S3 in the Supporting Information). This observation indicates that *trans-EE* is prone to form ternary complexes in the presence of DNA and Met-containing substrates, suggesting that this type of intermediate could play a major role in the cellular environment.

A contradictory role of Met was observed in the reaction of *cis*-DDP with GMP or oligomeric DNA. Met was found to increase the reaction rate of *cis*-DDP with GMP, suggesting that an intermediate Met adduct could transfer platinum onto DNA.[20] However, in the reaction with ss- or ds-DNA, Met and AcMet were found to decrease the DNA platination rate. Thus, it is rather unlikely that Met residues can favor DNA platination in the case of *cis*-DDP.[19]

Reactions of the *trans-EE*/AcMet complex with synthetic DNA oligomers and natural DNA were also carried out. The results of reactions with the ss-DNA pentamer d(TCGCT) and the palindromic ds-DNA dodecamer d(GATAGGCCTATC)$_2$ are reported in Figure 2. The ternary adduct with the

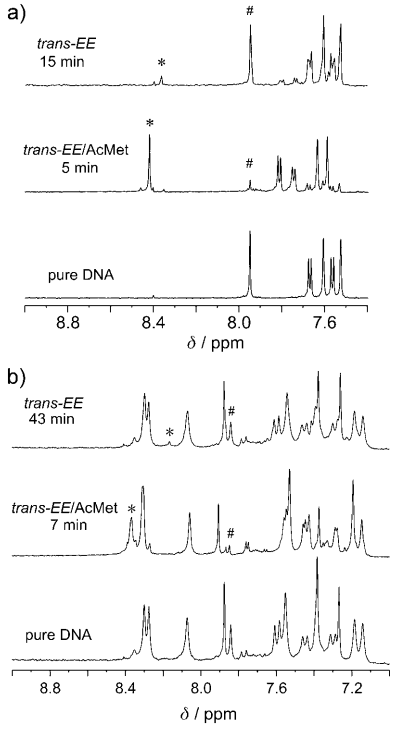

Figure 2. Aromatic region of 1D ^{1}H NMR spectra for the reaction of DNA with platinum complexes at 25 °C in 1:1 molar ratio (50 mM $NaClO_4$, 20 mM phosphate buffer, pH 7.0): a) ss-DNA pentamer d(TCGCT); b) ds-DNA dodecamer d(GATAGGCCTATC)$_2$. Platinum complex and reaction time are specified on the spectra. Symbols denote peaks from free DNA (#) and platinated DNA (*).

pentamer (DNA/*trans-EE*/AcMet) was purified by HPLC and its identity was confirmed by ESI-MS (m/z: calcd 991.192, obsd 991.180; Figure S4 in the Supporting Information). The ^{1}H NMR spectrum showed the typical 0.5 ppm downfield shift of the G–H8 signal, indicative of G–N7 platination (Figure 2a). As already observed in the case of GMP, the reaction of *trans-EE*/AcMet with the DNA pentamer was significantly faster than that of *trans-EE* (about 90% reaction after 5 min, compared with only 10% reaction after 15 min). Similar results were obtained in the case of the palindromic ds-DNA dodecamer d(GATAGGCCTATC)$_2$ (Figure 2b). This result represents a striking difference with respect to *cis*-DDP for which the adduct with a Met ligand reacted slightly faster with GMP but slower with DNA.

The rate of platination of natural DNA was monitored by fluorescence spectroscopy of the reaction of the *trans-EE*/AcMet complex with fish sperm DNA (Bio Basic Inc., High Purity). DNA exhibits a fluorescence band at 590 nm in the presence of a fluorescent probe such as ethidium bromide (EtBr). Platination of DNA reduces the fluorescence intensity and the decrease is proportional to the extent of platination.[21] By using this procedure, we evaluated the DNA platination rate by measuring the fluorescence decay as a function of time. Figure 3 shows that the platination of DNA

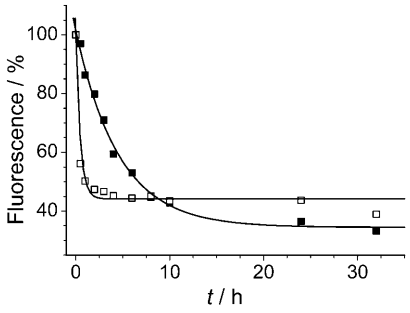

Figure 3. Fluorescence of fish sperm DNA upon reaction with *trans-EE* (■) and *trans-EE*/AcMet (□). The excitation wavelength was 530 nm and the emitted fluorescence was recorded at $\lambda = 592$ nm. Experimental conditions: 0.01 mg mL^{-1} DNA, 0.22 [Pt]/[Base] ratio, 10 mM phosphate buffer, 10 mM $NaClO_4$, and pH 7.0. Ethidium bromide (0.01 mg mL^{-1}) was added before measurement of the fluorescence.

by *trans-EE*/AcMet reached equilibrium after 1 hour, which is over one order of magnitude faster than the analogous reaction between DNA and *trans-EE*. These experimental data could be fitted with first-order kinetics and gave the pseudo-first-order rate constant $k = 5.8 \times 10^{-4}\ s^{-1}$ ($t_{1/2} =$ 20 min) for *trans-EE*/AcMet and $k = 6.1 \times 10^{-5}\ s^{-1}$ ($t_{1/2} =$ 190 min) for *trans-EE*. The platination of natural DNA by *trans-EE*/AcMet is significantly slower than that of monomeric or oligomeric nucleotides, probably because of folding, which limits the access of the platinum complex to the reactive sites of DNA. The amount of platinum loaded on DNA was measured by atomic absorption spectrophotometry, which confirmed a faster platination rate for *trans-EE*/AcMet than for *trans-EE* (data not shown).

The progress of the reaction of *trans-EE*/AcMet with GMP as a function of time at various pH values was also investigated (Figure S5 in the Supporting Information). Surprisingly, the reaction rate was significantly enhanced in acidic conditions. GMP was completely platinated by *trans-EE*/AcMet within 3 min at pH < 5.7, which is in accord with a value of $t_{1/2} \ll 1$ min. The pH dependence is likely to be related to the protonation status of *trans-EE*/AcMet, which may influence the reactivity with DNA; however, a detailed mechanistic investigation of the rate dependence upon pH was beyond the scope of this work. Tumor cells can have different acidity (pH_e in the range 5.7–7.8) depending upon the tumor types, whereas the pH_e value for normal cells remains steady at 7.4.[22] The higher reactivity of the *trans-EE*/Met adducts in acidic conditions could explain the different activity of the drug towards tumors of various types. In all living organisms, there are sulfur donors both in the extracellular and intracellular environment, and tumor cells of lower pH could therefore be more sensitive to the drug.

It can be envisaged that the strong *trans* effect of sulfur-donor ligands is responsible for the enhancement of the DNA platination rate in the case of the *trans-EE*/AcMet complex. Cisplatin and its analogues have two leaving groups in *cis* positions, so a sulfur-donor that has replaced the first leaving group can have little or no effect on the reactivity of the second leaving group. Moreover, it has been observed that the binding of Met to *cis*-DDP activates the ammine in the *trans* position,[20] and that the Met-rich motifs of CTR1 release all the ammine ligands from *cis*-DDP.[15] Therefore, S-containing molecules could play a completely different role in the reaction with DNA of platinum drugs with *cis* or *trans* geometry.

We investigated whether the formation of adducts between *trans-EE* and Met-containing peptides could be detected in cell systems. NMR spectroscopy can still be used for such an investigation if cells are supplemented with methyl-^{13}C L-Met (^{13}C Met). If a Met-containing protein is overexpressed, its interaction with the drug can also be monitored. The human protein ubiquitin (hUb) is particularly suitable for this purpose as it contains only one Met group and the SCH_3 group is hydrogen-bonded to the backbone NH site of Lys 63 and has a chemical shift that is distinct from those of other Met-containing peptides (Figure 4).

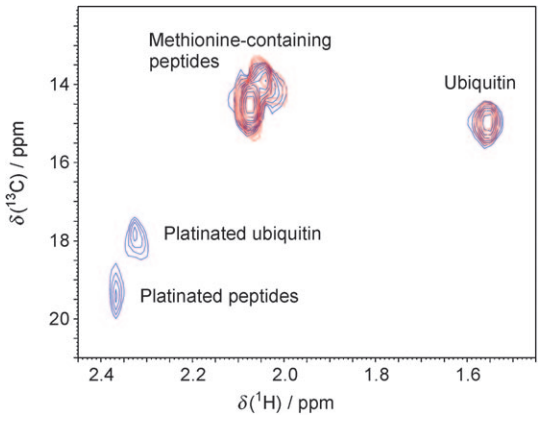

Figure 4. Overlay of ^{1}H,^{13}C HSQC spectra showing ^{13}C Met cross-peaks of extracts obtained from *E. coli* cells without (red contours) and after treatment for 3 h with *trans-EE* (blue contours).

E. coli cells overexpressing hUb selectively labeled with ^{13}C Met were incubated for 3 h at 37 °C with 1 mM *trans-EE*, washed, and resuspended in cell culture medium/sterile water (1:1). NMR analysis clearly shows the formation of *trans-EE*/Met adducts with appearance of a new $^{1}H/^{13}C$ cross-peak at lower field ($\delta = 2.37/19.4$ ppm) than that of unplatinated peptides (Figure 4). The $^{1}H/^{13}C$ shifts were very similar to those previously observed for platination of a Met-rich motif of CTR1.[15] In intact cells, the cross-peaks of hUb are barely visible because of the large correlation times.[23] However, after cell lysis (see the Supporting Information), the hUb cross-peak appears very intense and is accompanied by a weaker signal (ca. 15% intensity) at $\delta = 2.32/17.9$ ppm which can be confidently assigned to *trans-EE*-bound hUb Met1.

The formation of *trans-EE*/Met adducts in concentrations well above the μM range, notwithstanding the competition with all other platinophiles present in the cellular system, indicates that indeed this type of intermediate can play a major role in the physiological environment.

In conclusion, we have observed that the rate of DNA platination by a *trans*-platinum complex, such as *trans-EE*, is significantly enhanced by previous Met coordination. This rate enhancement was observed with all types of nucleotides studied in this work, including monomers, ss and ds-oligomers, and natural DNA. Moreover, the platination rate was found to be pH-dependent, which could correlate with the different drug sensitivity of various tumors. The binding of Met-containing peptides to *trans-EE* was also shown to occur readily in cellular systems. Taken together, the present results clearly demonstrate that S-donor molecules can play completely different roles in the cellular processing of *trans*-platinum drugs compared with antitumor drugs with *cis* geometry.

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