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Kinetic study of the reaction between an antitumor ^{15}N labeled *trans*-platinum iminoether complex and GMP by [^{1}H, ^{15}N] HMQC NMR †

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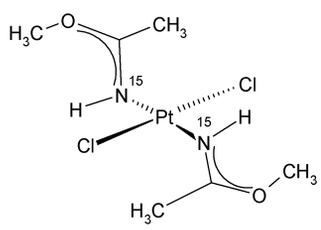
The kinetics and mechanism of binding of the ^{15}N-labeled anticancer compound *trans*-[$PtCl_2${*E*-HN=C(OMe)Me}$_2$] (*trans-EE*) to guanosine 5′-monophosphate (5′-GMP) and the tripeptide glutathione (GSH) have been investigated by 2D [^{1}H, ^{15}N] HMQC NMR spectroscopy. The different reaction products can easily be identified as crosspeaks in the 2D NMR spectra. The integrated peak intensities *vs.* time are used to obtain rate constants by a non-linear optimization procedure. The calculated rate constants (forward and back reaction) for hydrolysis of the first (k_1 and k_{-1}) and second chloride (k_2 and k_{-2}) are: $k_1 = 2.1 \times 10^{-4}$ s^{-1}, $k_{-1} = 890 \times 10^{-4}$ M^{-1} s^{-1}; $k_2 = 0.025 \times 10^{-4}$ s^{-1}, and $k_{-2} = 4.6 \times 10^{-4}$ M^{-1} s^{-1} at 298 K. The corresponding literature values for the inactive *trans*-[$PtCl_2(NH_3)_2$] compound exhibit a large dispersion. The formation of the mono-chloro GMP adduct takes place *via* a bimolecular process involving the mono-chloro mono-aqua species and the entering nucleotide ($k_3 = 2700 \times 10^{-4}$ M^{-1} s^{-1}), the concentration of this adduct reaches a maximum value after about 6 hours at 298 K. Subsequently, mono-aqua GMP ($k_4 = 0.131 \times 10^{-4}$ s^{-1}, $k_{-4} = 10.0 \times 10^{-4}$ M^{-1} s^{-1}) and bifunctional GMP adducts ($k_5 = 99.6 \times 10^{-4}$ M^{-1} s^{-1}) are formed in successive steps. Competition reactions of *trans-EE* with GSH and GMP show a clear preference for GSH adduct formation. Some of the reactions were run both with and without phosphate buffer to assess the degree of platinum–phosphate binding.

Introduction

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$] (*cis*-DDP), has been widely used in cancer chemotherapy for decades. The development of new platinum drugs still continues in order to provide agents which are less toxic than cisplatin and are active against different types of tumors. Since the *trans* isomer of *cis*-DDP is clinically ineffective it was assumed that the active platinum compounds had to be of the general form $PtAm_2X_2$ (Am_2 = two mono- or one bi-dentate amine ligands) with the two leaving groups X at the *cis* positions.[1] However, it has since been shown that several analogues of *trans*-DDP can exhibit antitumor activity comparable to that of *cis*-DDP.[2–5] Of particular interest is a group of iminoether derivatives of general formula *trans*-[$PtCl_2$(iminoether)$_2$] which was shown to be endowed with significant *in vivo* antitumor activity.[3–6] Surprisingly, these compounds were remarkably more cytotoxic than their *cis*-[$PtCl_2$(iminoether)$_2$] isomers.[2] Previous mechanistic studies concerned with the different activities of *cis*- and *trans*-DDP have focused on the lifetimes of their monofunctional adducts with DNA.[7] The half-lives for closure of monofunctional to bifunctional adducts have been reported to be 15 and 30 h for the *cis* and *trans* isomers, respectively.[8] For *trans*-[$PtCl_2$-{*E*-HN=C(OMe)Me}$_2$] (*trans-EE*) (the iminoether can have either *E* or *Z* configuration depending on the relative position of the alkoxy group and the N-bonded Pt atom with respect to the C=N double bond, only the *E* isomer has been considered in this investigation) a completely different mechanism seems to be responsible for the antitumor activity.[2,9,10] Compared to *cis*- and *trans*-DDP which have been shown to form bifunctional adducts with double-helical DNA, *trans-EE* forms kinetically stable monofunctional adducts at guanine residues of DNA.[6,11]



^{15}N labeled *trans-EE*

Previously, we have characterized a DNA duplex which was formed between 5′-(CCTCG*CTCTC) platinated by *trans-EE* at the G-residue and its complementary strand 5′-(GAGA-GCGAGG).[12] Surprisingly, this monofunctional binding by *trans-EE* was found to induce a 45° kink in the duplex comparable to the bending angle observed for bifunctional cisplatin adducts, however the bending was towards the minor groove.[9,13] Subsequent gel-electrophoretic studies by Brabec[14] showed that *trans-EE* induces approximately 30° bending of DNA at guanine residues in double-helical DNA. A detailed structural analysis of the adducts formed between *trans-EE* and GMP or AMP has been recently carried out based on NMR spectroscopy and molecular modeling.[15]

Other cellular biomolecules can compete with nucleotides for reaction with platinum, particularly cysteine containing peptides, due to the high affinity of platinum for S-donor ligands. Glutathione (γ-L-Glu-L-Cys-Gly, GSH), for example, has been shown to be kinetically more reactive than GMP even when the nucleotide is present in large excess.[16] GSH, methionine, and other sulfur-donor ligands have been found to play a role in the metabolism of cisplatin and, for example, [Pt(L-Met)$_2$] (mixture of *cis* and *trans* isomers) is a metabolite found in the urine of cisplatin-treated patients.[17]

† Electronic supplementary information (ESI) available: NMR spectra, differential equations and calculated curves for the reaction of *trans-EE* with GMP; competition between GMP and GSH for reaction with *trans-EE*. See http://www.rsc.org/suppdata/dt/b2/b203572n/

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In this paper we present a kinetic analysis of the reactions between *trans-EE* and GMP based on both HPLC and NMR data. The complex has been labeled with ^{15}N, which has allowed the use of two-dimensional [^{1}H, ^{15}N] HMQC (heteronuclear multiple quantum coherence) spectroscopy. The [^{1}H, ^{15}N] cross-peaks, which are diagnostic of the different species formed, are integrated and used as input in the kinetic analysis. In the presence of GSH, the reaction competition and the displacement of the platinum-coordinated nucleotides by the peptide have been studied. The results are discussed in relation to similar studies performed on *trans*-DDP in order to shed some light on the difference in antitumor activity between *trans-EE* and *trans*-DDP.

Experimental

Materials and sample preparation

The ^{15}N-labeled *trans-EE* complex was synthesized as previously described.[18] GMP and GSH were purchased from Sigma and used without further purification. Deuterium oxide was purchased from Fluorochem Limited Company.

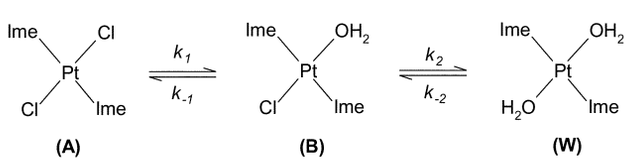
NMR spectroscopy

NMR spectra were recorded on a Bruker DRX 600 instrument operating at 600 Mz for ^{1}H. ^{1}H chemical shifts were referred to the H_2O signal (4.76 ppm at 298 K) and ^{15}N chemical shifts were referred to 1 M $^{15}NH_4Cl$ in 1 M HCl (external). All spectra were recorded at 298 K unless stated otherwise. Samples of *trans-EE* were dissolved in 0.5 ml 90% H_2O–10% D_2O containing 50 mM $NaClO_4$. No buffer was used in the GMP reaction with *trans-EE*; the initial pH was 6.0. A 40 mM phosphate buffer was used in the GSH reaction. NMR measurements of GSH reactions were performed in an Ar atmosphere. 1D ^{1}H spectra were typically recorded with a total of 32K complex points and 64–256 transients with 7200 Hz spectral width. The double pulsed field gradient spin echo (dpfgsew5) pulse sequence was employed to suppress water signals. 2 s relaxation delay was used and 0.3 Hz line broadening was added during the spectral processing. 2D [^{1}H, ^{15}N] HMQC NMR spectra[19] were acquired with ^{15}N decoupling. $^{1}J_{HN}$ was optimized at 78 Hz. The ^{1}H and ^{15}N spectral widths were set at 1216 Hz (^{15}N) and 6010 Hz (^{1}H) for the GMP reaction and at 2432 (^{15}N) and 12064 Hz (^{1}H) for the GSH reaction. The FID's were multiplied by a square-cosine function and zero-filled to 2048 complex points along F1 and F2.

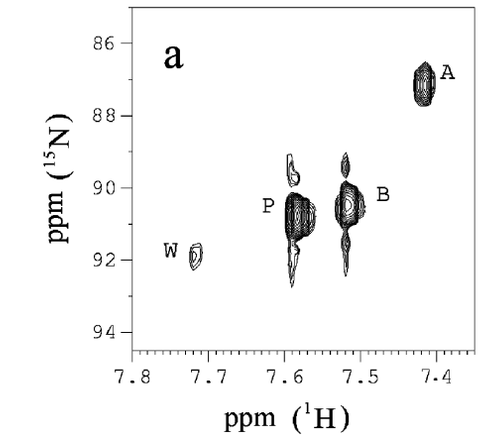
Results and discussions

Hydrolysis of *trans-EE*

The *trans-EE* hydrolysis was monitored by 2D [^{1}H, ^{15}N] HMQC spectra on ^{15}N labeled samples (Scheme 1). The two-dimensional spectrum after about 5 h reaction time (Fig. 1) shows the N–H crosspeaks assignable to the initial dichloro species (A) (7.42/87.28 ppm), the mono-chloro mono-aqua species (B) (7.52/90.50 ppm) and the di-aqua species (W) (7.72/91.9 ppm). Also a large crosspeak (P), corresponding to a platinum adduct with the phosphate buffer, is present. Replacement of Cl by H_2O causes a 1.4–3.2 ppm downfield shift of the iminoether ^{15}N signal and a 0.1–0.2 ppm downfield shift of the ^{1}H signal.



Scheme 1 Hydrolysis of *trans-EE* (Ime = iminoether ligand).



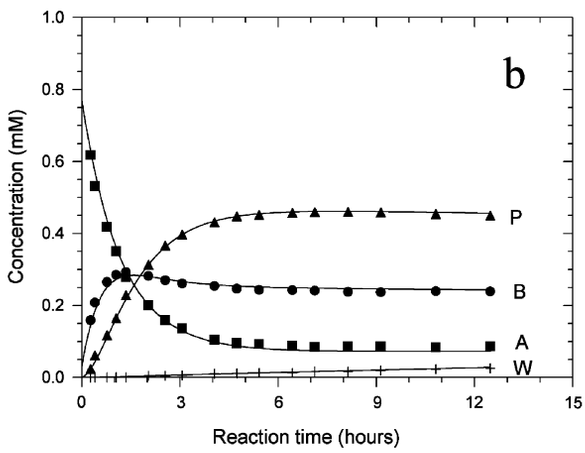


Fig. 1 Hydrolysis of 0.8 mM *trans-EE* in 40 mM phosphate buffer, pH 6.1, T = 298 K. (a) The two-dimensional [^{1}H, ^{15}N] HMQC spectrum was recorded 5 h after mixing of the reactants and shows N–H peaks for dichloro (A), chloro-aqua (B), diaqua (W), and phosphate (P) species. (b) Calculated curves and experimental NMR points for the time course of the hydrolysis reaction.

The rate constants for the hydrolysis were determined by a non-linear optimization procedure using the program SCIENTIST (version 2.01, MicroMath Inc.) and are reported in Table 1. According to the general trend in platinum(II) substrates, the rate constant for the first aquation process (k_1) is *ca.* 2 orders of magnitude greater than the rate constant for the second aquation process (k_2). Similarly the rate constant for the first anation reaction (k_{-1}) is *ca.* two orders of magnitude greater than the rate constant for the second anation reaction (k_{-2}). For both the k_1 and k_{-1} related processes the *trans* ligand (Cl^-) has a greater *trans*-labilizing effect than the *trans* ligand for the k_2 and k_{-2} related processes (H_2O). It is also to be noted that k_{-1} is *ca.* 400 times greater than k_1. The transition state is common to the two processes; therefore the greater activation energy for the forward with respect to the back reaction is to be associated with the greater stability of the dichloro with respect to the mono-chloro mono-aqua species. In a completely similar way k_{-2} is *ca.* 200 times greater than k_2, again the transition state is common to the two processes, therefore the greater activation energy for the forward with respect to the back reaction reflects the greater stability of the mono-chloro mono-aqua with respect to the di-aqua species.

A comparison with the corresponding rate constants for *trans*-DDP (Table 1) is not straightforward since published data for *trans*-DDP exhibit a large dispersion of values, probably because they have been determined under different experimental conditions and by different methods. An excellent review on the hydrolysis of *cis*- and *trans*-DDP has been published by Arpalahti.[20] It is expected that the rate constants for

Table 1 Rate constants for the hydrolysis reactions of *trans-EE* (this work), *cis*-DDP and *trans*-DDP (standard deviations in parentheses)

	trans-EE[a]	*cis*-DDP	*trans*-DDP
$k_1 \times 10^4$ s^{-1}	2.10(3)	0.518[b], 1.9(2)[d], 0.25[e],	0.19[b], 10.5(3)[d], 0.98[e],
$k_{-1} \times 10^4$ M^{-1} s^{-1}	890(5)	76.8[b], 62.6[c], 600(150)[d], 75.7[e],	305[b], 22000(2000)[d], 306[e],
$K_1 \times 10^4$ M	23.6	67.4[b], 31.6[d], 33[e],	6.22[b], 4.77[d], 3.2[e],
$k_2 \times 10^4$ s^{-1}	0.025(4)	0.25[b], 0.275[c], 2.3(3)[d], 0.33[e],	0.58[c], 0.04(2)[d], <0.5[e],
$k_{-2} \times 10^4$ M^{-1} s^{-1}	4.6(19)	909[b], 927[c], 9800(1400)[d], 825[e],	15100[c], 2000(200)[d],
$K_2 \times 10^4$ M	54.3	2.75[b], 2.97[c], 2.35[d], 4.0[e],	3.86[c], 0.2[d],

[a] 298 K, 0.05 M $NaClO_4$, this work. [b] 298 K, $I = 0.1$ M, ref. 21. [c] 298 K, $I = 1$ M, ref. 21. [d] 318.2 K, $I = 0.1$ M, ref. 23. [e] 298 K, $I = 0.318$ M, ref. 24.

solvolysis of the first chloride and for the back reaction (k_1 and k_{-1}, respectively) are faster for the *trans* isomer than for the *cis* isomer since the *trans* ligand in the substitution process has a greater *trans*-labilizing effect in the former case (Cl) than in the latter case (NH_3). In contrast the rate constants for hydrolysis of the second chloride and for the back reaction (k_2 and k_{-2}, respectively) are expected to be slower for the *trans* isomer than for the *cis* isomer, since the *trans* ligand in the former case (OH_2) has a smaller *trans* labilizing effect than the *trans* ligand in the latter case (NH_3). However, the values published by Miller *et al.*[21] and Hindmarsh *et al.*[22] show an opposite trend with the first hydrolysis of the *cis* isomer being faster than that of the *trans* isomer. We think that this is due to methodological problems. The authors used the spectral changes at specific isosbestic points to follow the two reactions separately with the assumption that the second Cl^- anation step is faster than the first ($k_{-2} \gg k_{-1}$). This procedure probably works with *cis*-DDP, where the assumption may be valid, but not with *trans*-DDP for which the second anation reaction (displacement of an aqua ligand *trans* to another aqua ligand) is expected to be slower than the first (displacement of an aqua ligand *trans* to chloro). Moreover they used an indirect detection method (based on the anation reactions) for monitoring the hydrolysis reaction and the strongly reversible nature of the first aquation step for *trans*-DDP may have caused additional problems. Also for *trans-EE* the first anation step has been found to be much faster than the second anation step (Table 1).

It is interesting to notice that in the *trans-EE* iminoether complex the equilibrium constant for the first solvolysis reaction is nearly one order of magnitude larger than for *trans*-DDP and quite close to that of *cis*-DDP. This observation supports the view that the difference in antitumor activity between *cis* and *trans* isomers may be due to kinetic effects. Furthermore, most data indicate that in the case of *cis*-DDP it is the mono-aqua species which makes the initial attack on DNA.[7,13]

Reaction of *trans-EE* with GMP

Both 1D ^{1}H and 2D HMQC NMR spectra were recorded for monitoring the reaction between ^{15}N labeled *trans-EE* and GMP. Fig. 2 shows a series of 1D ^{1}H spectra recorded at different time intervals. In the aromatic region, the H8 peak of free GMP gradually diminishes in intensity while three new H8 peaks emerge. The mono-adduct, represented by the new peaks C and D, is gradually converted to the bis-adduct represented by peak E which is the only major peak in the final spectrum. The same development can be observed for the *trans-EE* methyl and methoxy signals. These signals are in accordance with the ^{1}H NMR spectra of purified samples reported in previous work.[15] Minor peaks P and Q represent phosphate adducts which will be discussed below. The sensitivity of ^{1}H NMR is usually not sufficient for an accurate determination of rate constants.

The 2D [^{1}H ^{15}N] HMQC spectrum measured 6.5 h after mixing *trans-EE* and GMP is shown in Fig. 3. The ^{1}H–^{15}N cross-peaks representing the different species are all well separated and easily integrated. The five crosspeaks (A–E) are

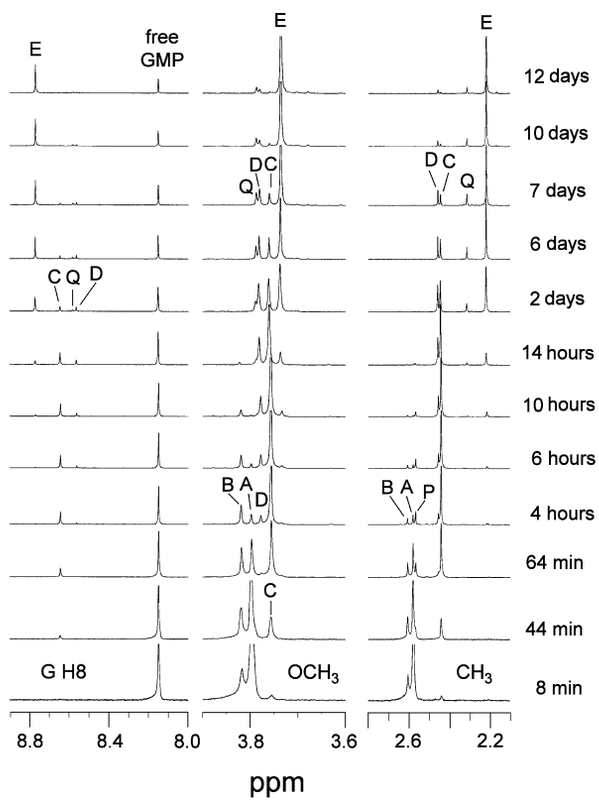


Fig. 2 Selected region of 1D ^{1}H NMR spectra for the reaction between 0.64 mM *trans-EE* and 1.8 mM GMP at various reaction times, 298 K, and pH 6.1 (50 mM phosphate buffer). Minor phosphate adducts are marked as P and Q.

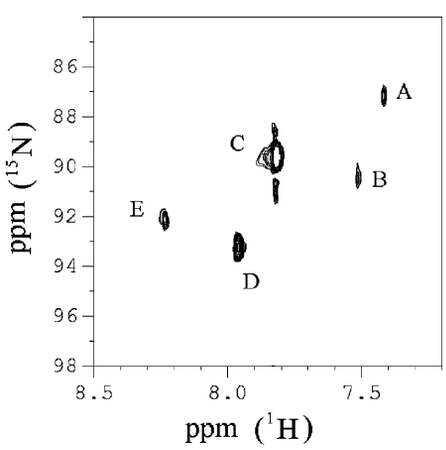


Fig. 3 2D [^{1}H, ^{15}N] HMQC spectrum for the reaction between 2.2 mM GMP and 0.9 mM *trans-EE*, 298 K, pH 6.1, recorded after 6.5 h reaction time.

assigned to: (A) *trans-EE* (Cl_2); (B) *trans-EE* (Cl, H_2O); (C) *trans-EE* (Cl, GMP); (D) *trans-EE* (H_2O, GMP); (E) *trans-EE* $(GMP)_2$ (Table 2). Replacement of Cl^- by water in adduct C to form adduct D leads to a ^{15}N downfield shift of 3.6 ppm, which

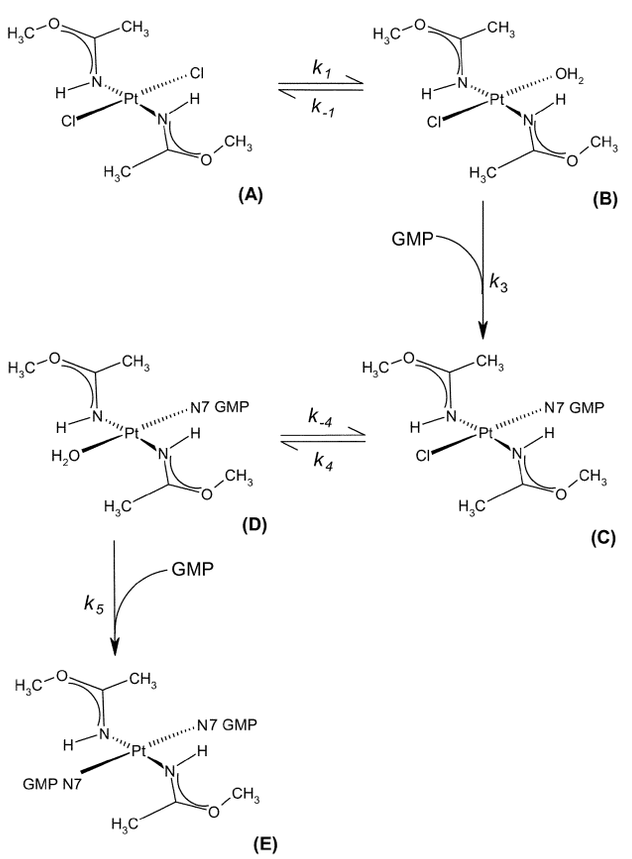
Table 2 ^{1}H and ^{15}N chemical shifts of HN at 298 K for *trans-EE* and its adducts

Compound	Peak	δ (^{1}H)	δ (^{15}N)
trans-EE (Cl_2)	A	7.42	87.2
trans-EE (Cl, H_2O)	B	7.52	90.5
trans-EE (Cl, GMP)	C	7.82	89.6
trans-EE (H_2O, GMP)	D	7.96	93.2
trans-EE $(GMP)_2$	E	8.23	92.1

is similar to the shift observed in the conversion of the chloro-aqua to the di-aqua species of *trans-EE*. The substitution of H_2O (O donor) by GMP (N donor) on going from B to C and from D to E leads to ^{15}N upfield shifts of 0.9 and 1.1 ppm, respectively. The di-aqua species observed in the solvolysis of pure *trans-EE* could not be observed in the reaction of *trans-EE* with GMP. The reaction was carried out at four different temperatures (288, 298, 308, and 318 K; Supplementary Material S3†).

If the reaction between *trans-EE* and GMP is carried out in the presence of phosphate buffer, two extra crosspeaks (P and Q) appear in the 2D HMQC spectrum (Fig. S1†). These two crosspeaks, appearing near to the peaks of the aqua species, were tentatively assigned to phosphate adducts.

2D [^{1}H, ^{15}N] HMBC spectra were also recorded based on NH/CH_3 $^{3}J_{HN}$ correlation (Supplementary Material S2†). The ^{3}J values observed for the different adducts were in the range 2.16–2.60 Hz and optimized at 2.3 Hz in the NMR measurements. HMBC (heteronuclear multiple bond correlation) spectra based on connectivities between ^{15}N and methyl ^{1}H provide additional support to the peak assignments. The HMBC results are less sensitive than HMQC; therefore only the HMQC data were used for the determination of rate constants according to Scheme 2. The crosspeak intensities *vs.* reaction time (Fig. 4) were fitted by numerical integration of a set of differential equations (Supplementary Material†) and the



Scheme 2

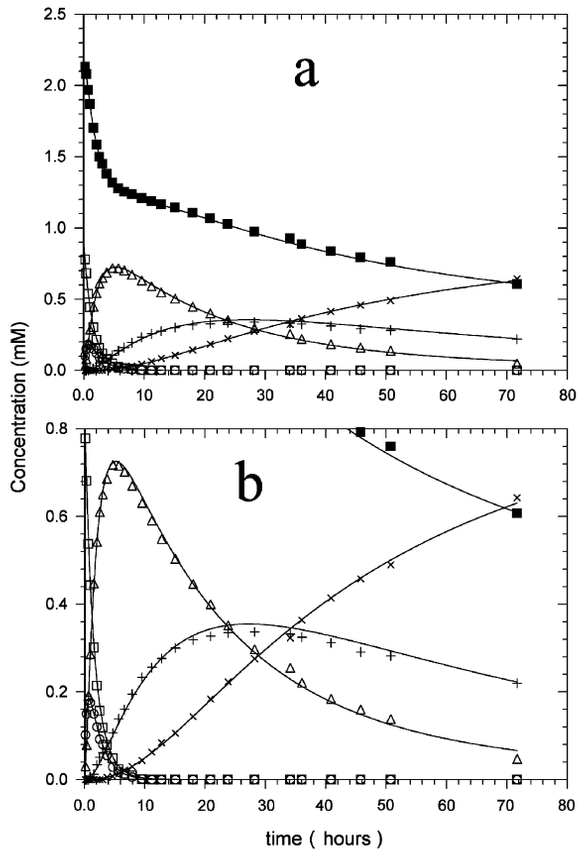


Fig. 4 Calculated curves and experimental NMR points for the time course of the reactions between *trans-EE* and GMP (b: expanded vertical scale). Symbols represent experimental data for: (■) GMP, (□) *trans-EE* (Cl_2), (○) *trans-EE* (Cl, H_2O), (△) *trans-EE* (Cl, GMP), (+) *trans-EE* (H_2O, GMP), (×) *trans-EE* $(GMP)_2$.

calculated rate constants are listed in Table 3. In order to gain further support for the second order mechanism of the platination steps (Scheme 2); additional experiments were performed in which the initial concentration ratio between *trans-EE* and GMP was changed (Supplementary Material S4†). In general the fitting between experimental points and calculated curves was good and therefore the calculated rate constants (Table 3) appear to be reliable.

A general assumption in substitution reactions in square-planar platinum(II) substrates is that the aqua species reacts fast with an incoming nucleophile. According to this assumption the rate of formation of C from A *via* the aqua species B (Scheme 2) should be independent of the concentration of GMP and the rate constant be equal to k_1 (s^{-1}). On the other hand the second-order process (rate constant = M^{-1} s^{-1}) in square-planar platinum(II) substrates usually accounts for the direct reaction between the unsolvated substrate and the incoming nucleophile. According to this general rule k_3 (M^{-1} s^{-1}) should represent the rate constant for the direct conversion of A into C and not of B into C (Scheme 2). Against this general rule is the evidence, shown in Fig. 3, that there is accumulation of the aqua species B. Therefore the transformation of B into C is not fast and the reaction rate may be dependent upon the GMP concentration. Moreover, since with the progress of the reaction there is accumulation of chloride ion which shifts the solvation equilibrium towards the dichloro species, the rate of formation of C is expected to decrease more rapidly with time if k_3 is the rate constant for the transformation of B into C than if k_3 were the rate constant for the transformation of A into C. The experimental data are in accord with the first alternative. Direct substitution pathways for the reactions A ⟶ C and C ⟶ E were also tested. The rate constants for these steps were found to be negligible: $k_{AC} = 3.2 \times$

Table 3 Rate constants for the reaction of *trans-EE* (this work) and *trans*-[$PtCl_2(NH_3)(c\text{-}C_6H_{11}NH_2)$] with GMP and of *trans*-DDP with inosine (standard deviations in parentheses)

	trans-EE[a] (298 K)	*trans-EE*[a] (308 K)	*trans*-$C_6H_{11}NH_2$ complex[b] (310 K)	*trans-EE*[a] (318 K)	*trans*-DDP[c] (318.2 K)
$k_1 \times 10^4$ s^{-1}	1.95(3)	5.0(1)	1.89(13)	11.1(3)	—
$k_{-1} \times 10^4$ M^{-1} s^{-1}	800(14)	810(340)	—	1300(100)	—
$K_1 \times 10^4$ M	24.4	61.7	—	85.4	—
$k_3 \times 10^4$ M^{-1} s^{-1}	2700(50)	3300(70)	1900(200)	7900(100)	14000(1000)
$k_4 \times 10^4$ s^{-1}	0.131(1)	0.518(7)	0.291(16)	1.60(2)	0.94(7)
$k_{-4} \times 10^4$ M^{-1} s^{-1}	10.0(14)	40(4)	—	160(10)	6200(1200)
$K_4 \times 10^4$ M^{-1} s^{-1}	131	130	—	100	1.52
$k_5 \times 10^4$ M^{-1} s^{-1}	99.6(9)	120(2)	430(40)	557(50)	3500(300)
k_5/k_3	0.0369	0.0364	0.226	0.0705	0.25

[a] 0.05 M $NaClO_4$, pH 6.1, this work. [b] GMP reaction, ref. 25. [c] I = 0.1M, inosine reaction ref. 23.

10^{-4} M^{-1} s^{-1} (0.12 % of k_3) and k_{CE} was too small to be measured. These results are in accordance with kinetic data for the reaction between *trans*-DDP and inosine nucleoside reported by Mikola and Arpalahti.[23]

It is interesting to notice that between k_4 and k_{-4} there is the same relationship (two orders of magnitude difference between the rate constants for the forward and back reaction) already observed for the first and second solvolysis of *trans-EE* in the absence of GMP (rate constants k_1 and k_{-1} and k_2 and k_{-2}, respectively). The same argument can be used for explaining the observed differences in the present case (different ground-state stability of the chloro-GMP with respect to the aqua-GMP species C and D, respectively). Moreover a factor of *ca.* 30 is found between k_3 and k_5. This difference is rather large, but still smaller than that observed between k_1 and k_2 for the solvolysis of *trans-EE* in the absence of GMP. Again the observed differences can be attributed to the different effect of the *trans* ligand in the substitution process which is Cl^- and GMP for k_3 and k_5, respectively, and Cl^- and H_2O for k_1 and k_2, respectively. The greater *trans* influence of GMP (N-donor) with respect to H_2O (O-donor) can explain why the difference is smaller between k_3 and k_5 than between k_1 and k_2.

As reported by Lippard and other authors, the rate-limiting step for initial binding of *cis*-DDP to DNA is the hydrolysis of the first chloride ion.[7] The rate constant for conversion of *trans-EE* dichloro to chloro-aqua species in the presence of GMP is $k_1 = 1.95 \times 10^{-4}$ s^{-1} as compared to $k_1 = 2.1 \times 10^{-4}$ s^{-1} in the absence of GMP. Also the equilibrium constants for the first hydrolysis step with and without GMP are, as expected, almost identical (Tables 1 and 3). Since we are not aware of kinetic data for the reaction of *cis*- and *trans*-DDP with GMP, we may compare our results with those of Arpalahti *et al.*[23] for the reaction between *trans*-DDP and inosine. Bearing in mind that nucleotides react appreciably faster than corresponding nucleosides, it is evident that mono-aqua *trans-EE* (Cl^-, H_2O) is much less reactive towards GMP than mono-aqua *trans*-DDP (Cl^-, H_2O).[20] We may also make comparison with the reaction between the *trans* isomer of JM118 (*trans*-[$PtCl_2(NH_3)(NH_2\text{-}C_6H_{11})$]) and GMP (Table 3). The rate constants for the *trans*-[$PtCl_2(NH_3)(NH_2C_6H_{11})$]/GMP reaction is, as expected, similar to that of *trans-EE*/GMP.[25] In this respect one may notice that the first hydrolysis step for *trans*-[$PtCl_2(NH_3)(NH_2C_6H_{11})$] was found to be *ca.* 3 times faster than for JM118 (*cis*). This is explained by the higher *trans* effect of Cl^-. The rate constant (k_4) for the second aquation step (Scheme 2) is comparable in magnitude for *trans-EE* and *trans*-[$PtCl_2(NH_3)(NH_2C_6H_{11})$].

Another feature of the *trans-EE* reaction is that the formation of the bis-adduct is quite slow in comparison to similar rate constants for other platinum complexes (Table 3). The k_5/k_3 value is 0.0369 for *trans-EE* at 298 K, compared to 0.25 and 0.226 for *trans*-DDP and *trans*-[$PtCl_2(NH_3)(c\text{-}C_6H_{11}NH_2)$], respectively. This result supports the previous observation by Brabec *et al.*[11] that DNA monofunctional *trans-EE* adducts are quite stable. One may conclude from these results that the second chloride ligand in *trans-EE* monofunctional adducts is not easily displaced. This is a significant feature because the monofunctional adducts of *trans-EE* were considered to be the active form in inducing cytotoxicity.

Table 4 Activation parameters for different reactions of *trans-EE* and *trans*-DDP

Reactant	Entering group	$\Delta H^{\neq}$/kJ mol^{-1}	$\Delta S^{\neq}$/ J mol^{-1} K^{-1}
A	H_2O	71.0	−75.1
B	GMP	38.4	−124.2
C	H_2O	96.5	−10.6
D	GMP	66.5	−58.5
trans-DDP[a]	H_2O	92.2	−26
trans-DDP[b]	H_2O	84	−46

[a] Ref. 21. [b] Ref. 7.

Determination of activation parameters

In order to provide further mechanistic insight into the reactions of *trans-EE* with GMP, the rate constants were determined at various temperatures in the range 288–318 K and the enthalpy ($\Delta H^{\neq}$) and entropy ($\Delta S^{\neq}$) were calculated from linear least-square fits of $\ln(k/T)$ *vs.* $1/T$ (Fig. 5).[26] The activation

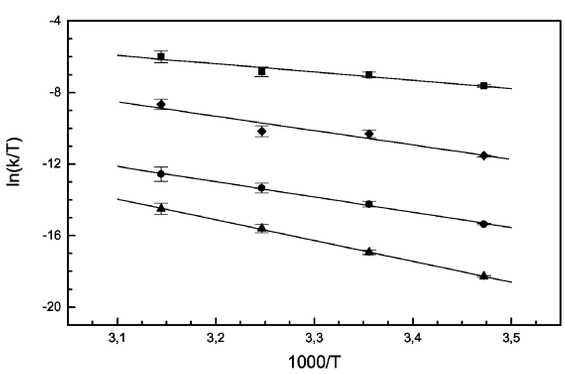


Fig. 5 Plot of $\ln(k/T)$ *versus* $(1/T)$. Symbols indicate the values for (●) k_1, (■) k_3, (▲) k_4, and (◆) k_5. The error bars correspond to 3 standard deviations.

parameters obtained are comparable to those reported for similar reactions involving *trans*-DDP (Table 4). The low enthalpies and negative entropies are indicative of an associative mechanism as generally found for substitution in square planar platinum complexes.[27]

Two new peaks appeared in the HMQC spectrum recorded at the highest temperature (318 K) after 23 hours of reaction and may be associated with a conformational change of the iminoether ligands. It was observed previously that the bis-adduct of *trans-EE* is slowly converted from *EE* to *EZ* at room temperature.[15] The crosspeak (*) appearing near peak E is

assigned to the *E* configuration whereas the crosspeak (#) outside the normal *trans-EE* region is assigned to the *Z* form (Fig. 6).

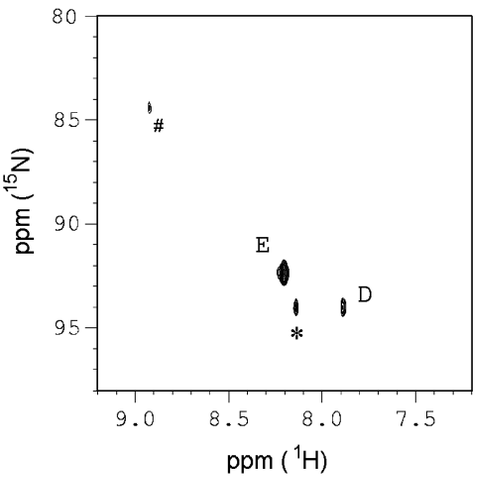


Fig. 6 2D [^{1}H, ^{15}N] HMQC spectrum for the reaction between 2.2 mM GMP and 0.9 mM *trans-EE* at 318 K, pH 6.1, recorded after 23 h reaction time. Two peaks labeled * and # are new peaks which were not observed in the reaction at lower temperatures.

Competition reactions of *trans-EE* with GSH and GMP

Reactions between *trans-EE* and GSH were monitored by [^{1}H, ^{15}N] NMR spectroscopy both with and without GMP. Fig. 7 shows the spectra recorded for the reaction between *trans-EE* and GSH after 29 minutes and 42 hours, respectively. Peaks marked A and B in Fig. 7a, are assigned to the dichloro and chloro-aqua *trans-EE* species. Peaks H–L represent different *trans-EE*/GSH adducts. The results show that, under the same experimental conditions, GSH reacts faster than GMP with *trans-EE* ($t_{1/2}$ of 19 and 65 min., respectively).

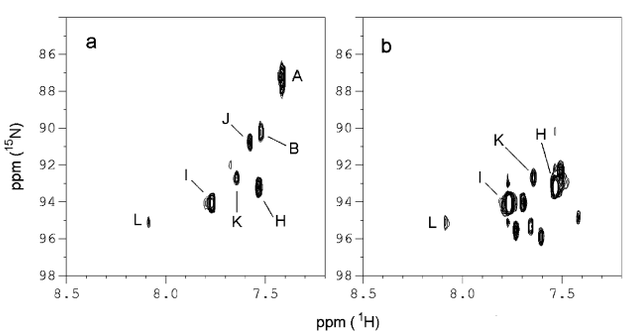


Fig. 7 2D [^{1}H, ^{15}N] HMQC spectra for reaction of 0.9 mM *trans-EE* with 2 mM GSH at 298 K. (a) 29 min reaction time, (b) 42 h reaction time. The peaks marked A and B are assigned to dichloro and chloro-aqua *trans-EE*, peaks H–L represent different *trans-EE*/GSH adducts.

The reaction between GSH and *trans-EE* was subsequently carried out in the presence of GMP. A mixture of *trans-EE*, GMP and GSH (0.9 : 2 : 2 molar ratios) was prepared and the reaction followed by 2D [^{1}H, ^{15}N] NMR. Spectra recorded after 1 h and 6.5 h are shown in Fig. 8. Plots of concentrations against time are shown in Fig. S6. † Comparison with the spectra obtained in the reactions of *trans-EE* with individual GMP or GSH allows the assignment of some of the crosspeaks. The dichloro (A) and mono-chloro mono-aqua (B) *trans-EE* adducts gradually disappear and peak (C), representing the *trans-EE*/GMP adduct, grows. *trans-EE* reacts with GSH forming several products (H–O). Some of these may be mixed-ligand adducts containing GMP *trans* to a GS^{-} ligand. Due to the complicated nature of GSH reaction products, it was not possible to assign all 17 peaks observed in this experiment. One new dominant peak (E) emerges in the 2D NMR spectra

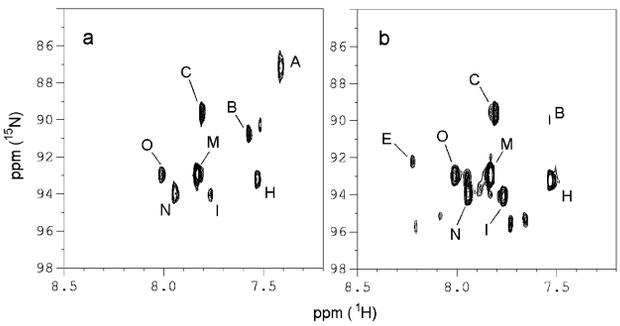


Fig. 8 2D [^{1}H, ^{15}N] HMQC spectra for reaction of 0.9 mM *trans-EE* with a mixture of 2 mM GMP and 2 mM GSH in phosphate buffer. (a) 1.0 h reaction time, (b) 6.5 h reaction time.

in almost identical position to the peak previously assigned to the bifunctional GMP/*trans-EE* adduct. Although formation of a bifunctional GMP adduct in the presence of GSH is generally considered unlikely, it has also been observed in a similar reaction involving *cis*-[$PtCl_2(NH_3)$(picoline)].[16a]

Platinum compounds usually react faster with GSH than with nucleotides because of the greater affinity of platinum for S- than for N-donors, and this has been demonstrated in competition reactions between $[PtCl(dien)]^{+}$ and GMP or GSH.[16c] However the reaction between the picoline anticancer complex *cis*-[$PtCl_2(NH_3)$(picoline)] and GSH was reported to be greatly influenced by the steric crowding of the ancillary ligands.[16a] The reduced reactivity with cellular thiols was considered responsible for the ability of this drug to circumvent drug resistance.[16c] The bulky iminoether ligands of *trans-EE* may produce the same effect in biological systems.

Conclusions

It has been postulated that, since *trans*-DDP is more reactive than *cis*-DDP, undesired reactions on its way to the pharmacological target may be responsible, at least in part, for lack of anticancer activity.[1] It has been suggested by Farrell *et al*.[28] that the anticancer activity of *trans* platinum complexes could be increased by sterically demanding carrier ligands which reduce the rate of replacement of the chloro ligands.

In this work we have shown that both the first hydrolysis step and the subsequent formation of the chloro-GMP adduct occur at a significantly slower rate for *trans-EE* than for *trans*-DDP. The rate constant for the first solvolysis step is one order of magnitude smaller for *trans-EE* than for *trans*-DDP and quite close to that of *cis*-DDP. This observation supports the suggestion that the difference in antitumor activity between *cis* and *trans* isomers is due to kinetic effects. Since most data indicate that the mono-aqua species is responsible for the initial attack on DNA, the search for new anticancer agents should be focused on fine-tuning of the reaction rates by proper choice of carrier ligands.

The same steric effects that slow down the rate of solvolysis may also prevent inactivation by sulfur containing biomolecules as shown by the formation of adducts of *trans-EE* with GMP even in the presence of glutathione.

Since it is evident that literature values for the rates of hydrolysis of *cis*- and *trans*-DDP exhibit a large dispersion having been measured under different reaction conditions, we plan to redetermine these values by using ^{15}N labeled Pt-compounds and [^{1}H, ^{15}N] NMR spectroscopy.

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References

1 B. Rosengerg, in *Cisplatin, Chemistry and Biochemistry of a Leading Anticancer Drug*, ed. B. Lippert, Wiley-VCH, Zurich, 1999, pp. 3–27.
2 G. Natile and M. Coluccia, in *Topics in Biological Inorganic Chemistry, Metallopharmaceuticals I, DNA Interactions*, ed. M. J. Clarke and P. J. Sadler, Springer-Verlag, Berlin, 1999, vol. 1, pp. 73–98.
3 M. Coluccia, A. Nassi, F. Loseto, A. Boccarelli, M. A. Mariggiò, D. Giordano, F. P. Intini, P. Caputo and G. Natile, *J. Med. Chem.*, 1993, **36**, 510.
4 N. Farrell, in *Metal Ions in Biological Systems*, ed. A. Sigel and H. Sigel, Marcel Dekker, Inc., New York, 1996, vol. 32, pp. 603–639.
5 L. R. Kelland, C. F. J. Barnard, I. G. Evans, B. A. Murrer, B. R. C. Theobald, S. B. Wyer, P. M. Goddard, M. Jones, M. Valenti, A. Bryant, P. M. Rogers and K. R. Harrap., *J. Med. Chem.*, 1995, **38**, 3016.
6 M. Coluccia, A. Boccarelli, M. A. Mariggiò, N. Cardellicchio, P. Caputo, F. P. Intini and G. Natile, *Chem.-Biol. Interact.*, 1995, **98**, 251.
7 D. P Bancoft, C. A. Lepre and S. J. Lippard, *J. Am. Chem. Soc.*, 1990, **112**, 6860.
8 J. L. Butour and N. P. Johnson, *Biochemistry*, 1986, **25**, 4534.
9 R. Zaludova, A. Zakovska, J. Kasparkova, Z. Balcarova, O. Vrana, M. Coluccia, G. Natile and V. Brabec, *Mol. Pharmacol.*, 1997, **52**, 354.
10 G. Natile and M. Coluccia, *Coord. Chem. Rev.*, 2001, **216–217**, 383.
11 V. Brabec, O. Vrana, O. Novakova, V. Kleinwachter, F. P. Intini, M. Coluccia and G. Natile, *Nucleic Acids Res.*, 1996, **24**, 336.
12 B. Andersen, N. Margiotta, M. Coluccia, G. Natile and E. Sletten, *Metal-Based Drugs*, 2000, **7**, 23.
13 E. R. Jamieson and S. J. Lippard, *Chem. Rev.*, 1999, **99**, 2467 and references therein.
14 V. Brabec, private communication.
15 Y. Liu, M. F. Sivo, G. Natile and E. Sletten, *Metal-Based Drugs*, 2000, **7**, 169.
16 (*a*) Y. Chen, Z. Guo, J. A. Parkinson and P. J. Sadler, *J. Chem. Soc., Dalton Trans.*, 1998, 3577; (*b*) R. N. Bose, S. Moghaddas, E. L. Weaver and E. H. Cox, *Inorg. Chem.*, 1995, **34**, 5878; (*c*) M. I. Djuran, E. L. M. Lempers and J. Reedijk, *Inorg. Chem.*, 1991, **30**, 2648.
17 R. E. Norman, J. D. Ranford and P. J. Sadler, *Inorg. Chem.*, 1992, **31**, 877.
18 R. Cini, P. Caputo, F. P. Intini and G. Natile, *Inorg. Chem.*, 1995, **34**, 1130.
19 A. G. Palmer, J. Cavanagh, P. E. Wright and M. Rance, *J. Magn. Reson.*, 1991, **93**, 151; L. E. Kay, P. Keifer and T. Saarinen, *J. Am. Chem. Soc.*, 1992, **114**, 10663; J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S. J. Glaser, O. W. Sorensen and C. Griesinger, *J. Biomol. NMR*, 1994, **4**, 301–306.
20 J. Arpalahti, in *Metal ions in Bioligical System*, ed. A. Sigel and H. Sigel, Marcel Dekker, Inc., New York, 1996, vol. 32, pp. 379–395.
21 S. E. Miller, K. J. Gerard and D. A. House, *Inorg. Chim. Acta.*, 1991, **190**, 135.
22 K. Hindmarsh, D. A. House and M. M. Turnbull, *Inorg. Chim. Acta.*, 1997, **257**, 11.
23 M. Mikola and J. Arpalahti, *Inorg. Chem.*, 1994, **33**, 4439.
24 F. Aprile and D. S. Martin, *Inorg. Chem.*, 1962, **1**, 551.
25 S. J. Barton, K. J. Barnham, U. Frey, A. Habtemariam, R. E. Sue and P. J. Sadler, *Aust. J. Chem.*, 1999, **52**, 173.
26 H. Eyring, *Chem. Rev.*, 1935, **17**, 65.
27 C. H. Langford and H. B. Gray, *Ligand Substitution Processes*, W. A. Benjamin, London, 1966, p. 43.
28 N. Farrell, T. T. B. Ha, J. P. Souchard, F. L. Wimmer, S. Cros and N. P. Johnson, *J. Med. Chem.*, 1989, **32**, 2240.