Novel Adducts of the Anticancer Drug Oxaliplatin with Glutathione and Redox Reactions with Glutathione Disulfide

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Reactions of [Pt(*rac*-1,2-DACH)Cl_2], [Pt(1*R*,2*R*-DACH)Cl_2] and the anticancer drug oxaliplatin with the tripeptide glutathione and glutathione disulfide have been studied by HPLC, ESI-MS and NMR methods. The same two major products are formed in each reaction: a thiolate-bridged dimer and a novel thiolate-bridged dinuclear macrochelate containing a nine-membered chelate ring. Redox reactions of Pt^{II} anticancer drugs with biological disulfides are of potential importance to their mechanism of action.

Introduction

cis-Diamminedichloroplatinum(II) or cisplatin (**1**) has been used as an anticancer drug since it entered clinical trials in the 1970s.[1] Its effectiveness against ovarian and testicular cancers is well established, and it has also been used to improve the response rate of head, neck and lung tumours.[2] Although one of the most effective anticancer drugs known to date, cisplatin possesses inherent disadvantages, namely limited solubility and extreme side-effects, including nausea, vomiting and nephrotoxicity.[3] Furthermore, many tumours have displayed a natural resistance to cisplatin while others develop resistance during treatment.[4] Thus, the use of the drug is limited to a narrow class of tumours. The occurrence of such adverse side-effects has been addressed by the introduction of hyperhydration therapies and serotonin antagonists.[5] Concurrently, there has been a major effort in the production of alternative drugs, which display all of the activity of cisplatin but with fewer side-effects.[6] A large library of platinum complexes has been synthesised and their antitumour activity examined, resulting in the determination of structure-activity relationships.

The nature of the amine has a pronounced effect on the activity observed. Preclinical studies of platinum complexes containing diaminocyclohexane (DACH) have demonstrated activity against cisplatin-resistant cell lines.[7] The drug (1*R*,2*R*)-diaminocyclohexaneoxalatoplatinum(II), oxaliplatin (**2**), has recently been approved for use in the treatment of colorectal and ovarian cancers in Europe, Asia and South America.[8,9] In the clinical trials of oxaliplatin, the drug was highly active in solid tumour types, and particularly effective against advanced colorectal cancer when administered with 5′-fluorouracil.[5,10] Oxaliplatin shows no nephrotoxicity or myeloid toxicity. However, the dose-limiting toxicity is governed by peripheral sensory neuropathy.[11] Evidence also exists that cisplatin analogues containing the bulky, nonpolar ligand DACH, such as oxaliplatin, do not elicit resistance in mismatch repair-deficient cells.[12] Thus, oxaliplatin may be able to overcome tumour resistance caused by loss of mismatch repair. Studies have shown that [Pt(1,2-DACH)Cl_2] is nearly twice as cytotoxic to HT-29 cells as oxaliplatin, although cellular uptake of oxaliplatin is considerably higher.[11]

Although cellular DNA is the ultimate target for platinum anticancer drugs,[13−15] many other biomolecules can also interact with platinum drugs, especially thiols and thioethers. Such biomolecules could either aid the transport of the drug or, equally, hinder transport by effectively capturing it, thereby preventing it from ever reaching its intended target site.

The thiol-containing tripeptide glutathione (γ-L-Glu-L-Cys-Gly, GSH, **3**) is present in most cells in millimolar concentrations (0.5−10 mM).[16,17] Intracellular interactions between glutathione and cisplatin have previously been found to be deactivating, and cisplatin-resistant tumour cells often contain elevated levels of glutathione.[18,19] There is strong evidence that GS-X pumps can facilitate the active transport of Pt drugs from the cell.[20] Interestingly, however, the strong affinity of sulfur for platinum complexes has led to the coadministration of platinum drugs with glutathione as a chemoprotectant.[21,22] The toxic side-effects are reduced due to reversal or prevention of Pt-S adducts in proteins, while the antitumour activity of cisplatin remains un-

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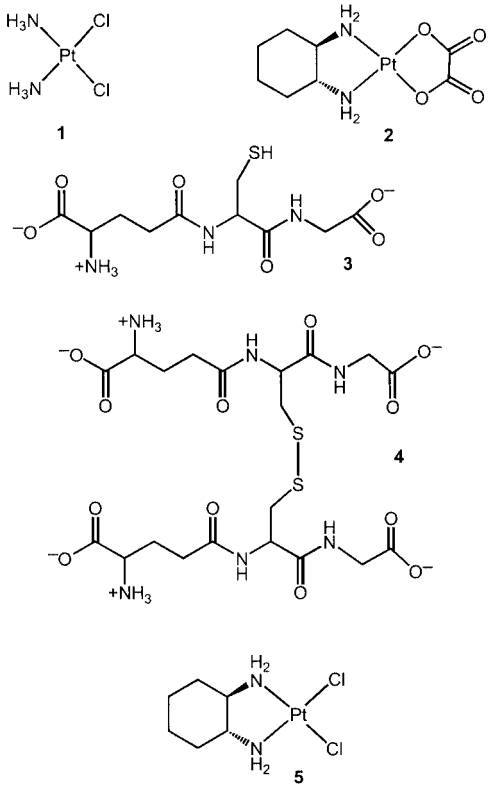
affected.[23] Platinum-sulfur adducts might constitute drug reservoirs from which the platinum could later be transferred onto the target DNA.[17] Could this provide an alternative pathway to the direct attack of the drug (or its aqua adducts) on DNA? Such a question has provided the stimulus for a number of studies into reactions between platinum drugs and potential sulfur nucleophiles.[24−28]

The major product from the reaction of cysteine derivatives with *cis*-diam(m)ineplatinum(II) complexes is thought to be the bridged dimer $[(am)_2Pt(\mu_2\text{-}SR)_2Pt(am)_2]$, where "am" is am(m)ine or one half of a diamine. However, these bridged complexes are unlikely to be reactive towards DNA bases, although the ammine complexes can slowly release ammonia.[29,30] Reactions carried out with [PtCl(dien)]Cl and methionine (Met) or methylated glutathione (GSMe)[26,27] and 5′ GMP have indicated that the platinum−sulfur adducts $[Pt(dien)(Met\text{-}S)]^{2+}$ and $[Pt(dien)(GSMe\text{-}S)]^{2+}$ are formed initially with subsequent intermolecular displacement of the thioether in the Pt−S adducts by 5′ GMP-N7. Interestingly, this displacement of guanine-N7 has been observed only with thioether adducts and not for reactions with thiolate complexes.[30] The biotransformation products of oxaliplatin have been investigated in patients with gastrointestinal cancers and there is evidence of nucleophilic substitution of the oxalate leaving group, with mono- and dichlorinated DACH-platin and glutathione-DACH-platin adducts being present as metabolites in the blood plasma and urine.[31]

In work reported here, we have isolated and characterised new products formed from reactions of either GSH or GSSG (GSH disulfide, **4**) with oxaliplatin, and (1*R*,2*R*)-diaminocyclohexanedichloroplatinum(II), [Pt(1*R*,2*R*-DACH)-Cl_2], **5**. These reactions have been carried out under physiological conditions, and for each complex a glutathione-bridged dinuclear platinum(II) macrochelate has been isolated. This is a potentially reactive species and could play a role in the mechanism of action of platinum anticancer drugs.



Results

Reactions of [Pt(1,2-DACH)Cl_2] with GSH and GSSG

UV/Vis Measurements

The time-courses of reactions between [Pt(*rac*-1,2-DACH)Cl_2] with GSH or GSSG at 310 K were studied using UV/Vis spectroscopy. Two molar equivalents of GSH or GSSG (10 μL, 20 mM, pH 7) were added to a solution of [Pt(*rac*-1,2-DACH)Cl_2] (1 mL, 100 μM). Typical spectra are shown in Figure 1. In both experiments a major new absorption band was observed at 220 nm which increased in intensity with time over a period of ca. 24 h. Interestingly, the reaction with GSH was only slightly faster than that with GSSG [see also Figure 8(b)].

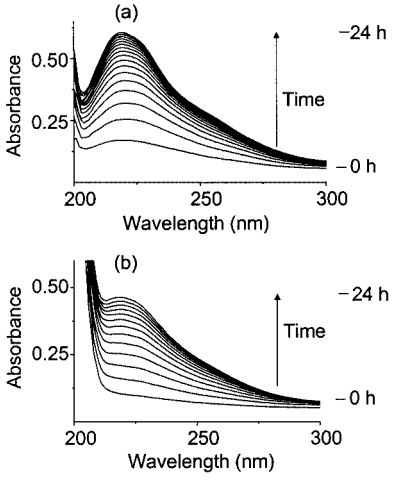


Figure 1. The change in absorbance with time for reactions of [Pt(*rac*-1,2-DACH)Cl_2] with two molar equivalents of (a) GSH, or (b) GSSG in aqueous solution (pH 7); measurements were taken at 310 K at regular hourly intervals for the first 24 hours of the reaction

HPLC Measurements

HPLC techniques were used to isolate two major products (labelled **A** and **B** in Figure 2) which formed during six-day incubations at 310 K of [Pt(*rac*-1,2-DACH)Cl_2] (2 mL, 100 μM) with two molar equivalents of GSH or GSSG (20 μL, 20 mM) initially at pH 7. Small amounts of unchanged GSH and GSSG were still present in the reaction mixture after six days. The major product peaks eluted at 20.6 min (peak **A**) and 21.6 min (peak **B**) using a gradient of acetonitrile (0−30% over 25 min) in water, with 0.1% trifluoroacetic acid as an ion pairing agent. Other minor peaks were also observed in the chromatogram, but these peaks were not isolated or identified.

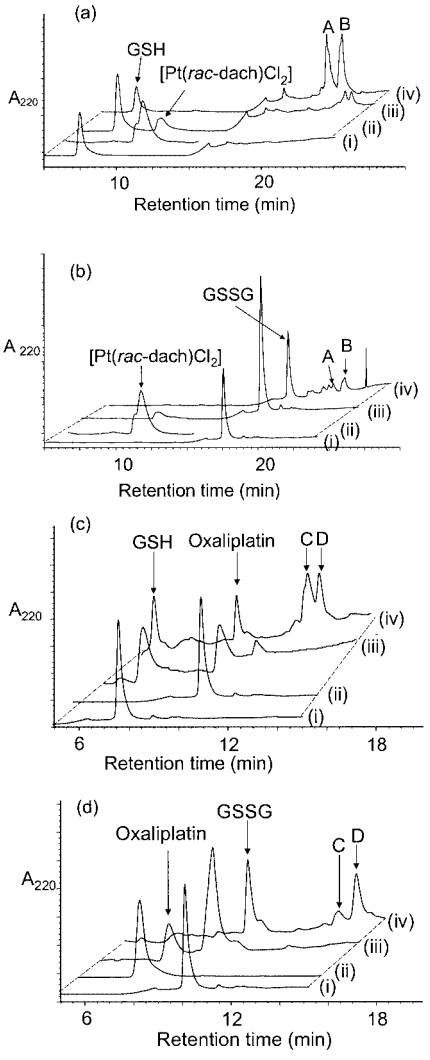


Figure 2. HPLC chromatograms (C_{18} reverse phase column eluted with a CH_3CN/H_2O gradient containing 0.1% of the ion pairing agent, monitored at 220 nm) for reactions of [Pt(*rac*-1,2-DACH)Cl_2] with two molar equivalents of either (a) GSH, or (b) GSSG (310 K, initially at pH 7), and oxaliplatin with two molar equivalents of either (c) GSH, or (d) GSSG (310 K, initially at pH 7); labels for chromatograms: (i) GSH or GSSG alone, (ii) [Pt(*rac*-1,2-DACH)Cl_2] or oxaliplatin alone (iii) after 5 min of reaction and (iv) after six days of reaction; baseline drifts in the chromatograms are observed due to a change in the refraction index of the solvent

Using the same acetonitrile gradient, the reaction of [Pt(*rac*-1,2-DACH)Cl_2] (2 mL, 100 μM) with two molar equivalents of GSH (20 μL, 20 mM, pH 7) at 310 K was monitored at hourly intervals for 24 h. The resulting chromatograms are shown in Figure S1 in the Supporting Information. The chromatograms show that the peaks for the starting materials decrease and the intensities of the product peaks **A** and **B** increase with time. No intermediates were observed during the course of the reaction. The reaction was complete after ca. 24 h, at which point the peaks due to the starting materials, 8.1 min (GSH) and 8.9 min ([Pt(*rac*-1,2-DACH)Cl_2]), have almost disappeared and the peaks for the products, 19.1 min (**A**) and 22.1 min (**B**), reach maximum intensity.

Reactions of [Pt(*rac*-1,2-DACH)Cl_2] with increasing GSH concentrations (1, 2, 4, 8 and 10 molar equivalents) were investigated by HPLC using a different acetonitrile gradient (4−20% over 30 min). The resulting chromatograms are shown in Figure 3. With this acetonitrile gradient the peaks that represent the major products, peaks **A** and **B**, do not appear as the single peaks previously seen in Figure 2, but as a series of split peaks. This difference can be attributed to changes in the HPLC elution conditions and the possible presence of isomers. The chromatograms clearly indicate a steady increase in the area of the peaks for product **A** (ca. 14 min) with increasing concentrations of GSH, while the intensities of the peaks for product **B** (ca. 18 min) decrease with increasing GSH concentrations. The areas of the peaks for products **A** and **B** are approximately equal at a 2:1 molar ratio of GSH to [Pt(*rac*-1,2-DACH)Cl_2].

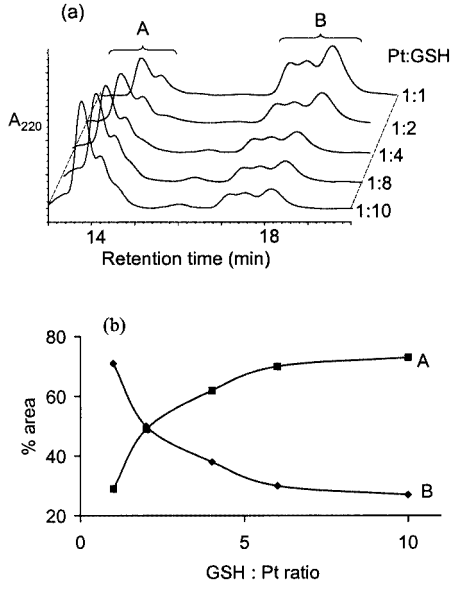


Figure 3. (a) Effect of variation of the molar ratio of [Pt(*rac*-1,2-DACH)Cl_2]:GSH on the intensities of the groups of peaks **A** and **B** in the HPLC chromatogram from reactions incubated at 310 K for 6 days; (b) variation of the intensities of peaks **A** and **B** with Pt:GSH molar ratio

To investigate the possibility that the split peaks observed in the HPLC chromatograms shown in Figure 3 are due to the presence of isomers, an analogous reaction of GSH (20 μL, 20 mM, pH 7) with enantiomerically pure [Pt(1*R*,2*R*-DACH)Cl_2] (2 mL, 100 μM) was carried out at 310 K. The resulting HPLC chromatogram, obtained using a 4−20% acetonitrile gradient over 30 min, is shown in Figure 4, together with the HPLC trace of products from the reaction of [Pt(*rac*-1,2-DACH)Cl_2] and GSH for comparison. The chromatogram for the reaction products of the enantiomerically pure platinum complex contains two single peaks in a ratio of ca. 1:2. The overall ratio of peak areas **A**:**B** is similar for reactions of the racemic and enantiomerically pure DACH complexes. The small change in HPLC retention times can be attributed to slight differences in the HPLC elution conditions.

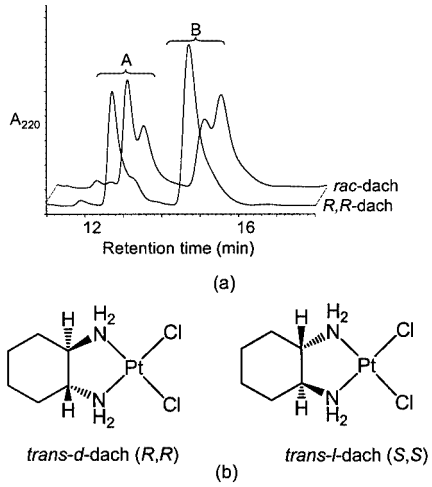


Figure 4. (a) HPLC chromatograms for products formed at 310 K from the reaction of [Pt(*rac*-1,2-DACH)Cl_2] or [Pt(1*R*,2*R*-1,2-DACH)Cl_2] with two molar equivalents of GSH in aqueous solution (initially at pH 7); HPLC elution conditions are slightly different from those in Figures 2 and 3, accounting for slight variations in the retention times; (b) structures of the two optical isomers of [Pt(1,2-DACH)Cl_2], [Pt(1*R*,2*R*-DACH)Cl_2] and [Pt(1*S*,2*S*-DACH)Cl_2]

The absorption coefficients of the separated products **A** (ε = 32,000 $M^{-1}cm^{-1}$) and **B** (ε = 34,000 $M^{-1}cm^{-1}$) at 220 nm were determined by freeze-drying the collected peaks, redissolution in water, measurement of their absorption spectra and determination of the platinum concentration in the same solution by ICP-AES.

Since GSH in cells is likely to be present in high molar excess over the platinum complex, an analogous reaction of [Pt(1*R*,2*R*-DACH)Cl_2] and a 100-fold molar excess of GSH was also monitored by HPLC. Two product peaks were formed in this reaction, and their identities (peaks **A** and **B**) were confirmed by ESI-MS (see below).

ESI-MS Measurements

The positive ion ESI-MS data of the reaction mixtures of [Pt(*rac*-1,2-DACH)Cl_2] with GSSG or GSH revealed the presence of peaks for two major products at *m*/*z* = 462 and *m*/*z* = 615 for both of the reactions (Figure S2 in the Supporting Information). The only other observed peaks are attributable to small amounts of the starting materials. Peaks **A** and **B** were separated by HPLC, and ESI-MS measurements of the collected fractions indicated that each peak consists of a single product. The observed ESI-MS spectrum for each of these products is shown in Figure 5. Both peaks show a cluster of ions characteristic of the isotopic distributions of Pt and N. The most abundant molecular ion obtained from collected peak **A** has a molecular ion [M^{2+}] at *m*/*z* = 615. Isotope modelling suggested that peak **A** corresponds to the molecular formula of $[Pt_2C_{32}H_{58}N_{10}O_{12}S_2]^{2+}$, assignable to an *S*-bridged dimer [Figure 6(a)]. The fraction associated with peak **B** [Figure 5(b)] has a molecular ion [M^{2+}] at *m*/*z* = 462, and isotope modelling suggested a molecular formula

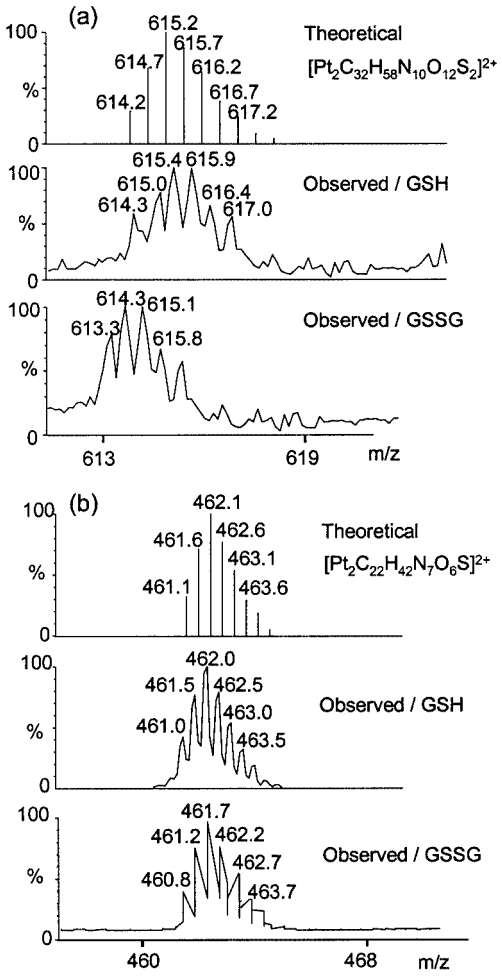


Figure 5. Positive ion ESI-MS spectra for HPLC peaks (a) **A** and (b) **B** separated from the reaction of [Pt(*rac*-1,2-DACH)Cl_2] with two molar equivalents of GSH or GSSG (310 K, initially at pH 7, six days); the HPLC conditions for the separation of these species are the same as in Figure 2; the HPLC peaks were collected, concentrated by lyophilisation and redissolved in acetonitrile/water, 1:1, for ESI-MS measurements; the observed and theoretical isotope patterns of the proposed structures for each peak are shown; the full ESI-MS spectrum is shown in Figure S2 in the Supporting Information

$[Pt_2C_{22}H_{42}N_7O_6S]^{2+}$, assignable to a glutathione-bridged macrochelate [Figure 6(b)].

NMR Analysis

1D ^1H and 2D ^1H DQFCOSY, NOESY, TOCSY and ROESY NMR experiments were carried out on a ca. 1.3 mM solution of product **B** in 90% H_2O/10% D_2O, pH 4. Specific assignments were determined for most of the ^1H NMR resonances based on the 2D TOCSY NMR spectroscopic data (Figure 7): Gly-N*H* (δ = 7.90 ppm) couples to two doublets of doublets at δ = 4.10 ppm (Gly−Hα^a) and δ = 3.61 ppm (Gly−Hα^b). Cys−Hα (δ = 5.02 ppm) couples to Cys−Hβ^b (δ = 2.79 ppm) but not to its geminal partner Cys−Hβ^a (δ = 2.66 ppm). Both of these signals are clearly visible in the 1D ^1H NMR spectrum (Figure S3 in the Supporting Information, which also shows the atom labelling scheme). A spin system consisting of six resonances was

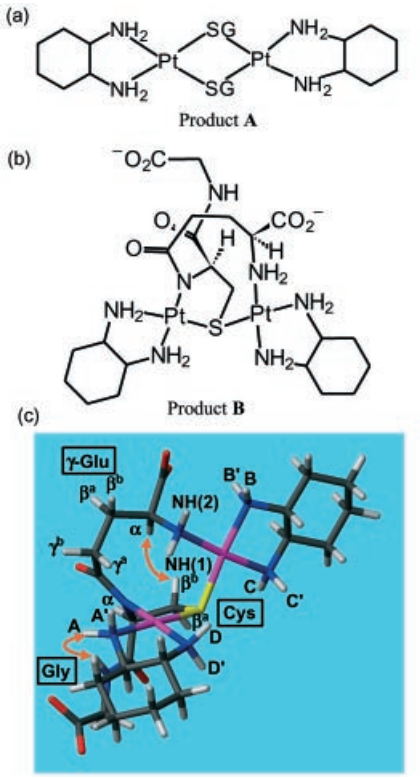


Figure 6. Proposed structures for the products of the reactions of [Pt(*rac*-DACH)Cl_2] with GSH or GSSG, which give rise to HPLC peaks (a) **A**, and (b) **B**; (c) capped-stick representation of the structure of the product corresponding to HPLC peak **B** formed between GSH with [Pt(1*R*,2*R*-DACH)Cl_2], as determined by ^{1}H NMR spectroscopy; two key structural ^{1}H-^{1}H rOes are shown by double-headed arrows; of the N*H* signals, the highest frequency resonance is that of NH(1) of γ-Glu; the space-filled representation explains how this shift may arise since the proton sits above one of the platinum centres (see contents entry); this proton therefore experiences a larger deshielding effect compared to all other N*H* protons in the molecule

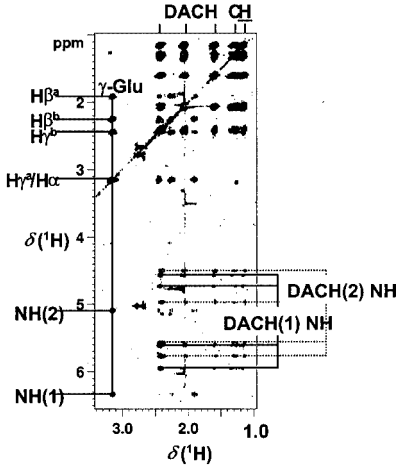


Figure 7. Section of the 600 MHz ^{1}H 2D TOCSY NMR spectroscopic data for peak **B** formed between GSH with [Pt(1*R*,2*R*-DACH)Cl_2], showing assignments for γ-Glu and DACH ligand resonances; despite several overlaps between DACH C*H* protons, it is still possible to distinguish two independent spin systems from the DACH N*H* resonances; resonance assignments are summarised in Table 1

assigned to γ-Glu: $\delta_{NH(1)}$ = 6.33 ppm; $\delta_{NH(2)}$ = 5.10 ppm; $\delta_{H\alpha}$ = 3.15 ppm; $\delta_{H\beta}{}^{b}$ = 2.25 ppm; $\delta_{H\beta}{}^{a}$ = 1.91 ppm; $\delta_{H\gamma}{}^{a}$ = 3.25 ppm; $\delta_{H\gamma}{}^{b}$ = 2.40 ppm. In addition, two complete spin systems are visible for two independent DACH units (Figure 7). Stereospecific assignments for the ring protons were not made due to resonance overlap. The NMR signal assignments are given in Table 1.

Table 1. ^{1}H NMR resonance assignments for the 2:1 macrochelate (peak **B**) formed between [Pt(1*R*,2*R*-DACH)Cl_2] and GSH; for proton labelling see Figures 6(c) and Figure S3 in the Supporting Information

		δ (NH)				
{Pt-DACH}		5.95 (A), 4.56 (A′) 5.62 (D), 4.75 (D′)			5.78 (C), 4.96 (C′) 5.57 (B), 4.50 (B′)	
H_2-SG	δ (NH)	δ (Hα)	δ (Hβ^a)	δ (Hβ^b)	δ (Hβγ^a)	δ (Hβγ^b)
Gly	7.90	4.10[a], 3.61[b]				
Cys	– [a]	5.02	2.66[b]	2.79[c]		
γ-Glu	6.33 (NH1), 5.10 (NH2)	3.15	1.91	2.25	3.16	2.40

[a] The NH signal is absent, consistent with the N of Cys being bound directly to one of the two platinum metal centres. [b] $^3J_{H,H}$ = 0, $^2J_{H,H}$ = 14 Hz. [c] $^3J_{H,H}$ = 6.4, $^2J_{H,H}$ = 14 Hz.

A molecular model of product **B** was constructed as a glutathione-bridged macrochelate molecule, $[Pt_2C_{22}H_{42}N_7O_6S]^{2+}$, which accounts for three specific observed rOes: Gly-N*H* shows an rOe to the NH proton A of DACH(1); Cys-Hβ^b shows an rOe to γ-Glu−Hα; γ-Glu−Hγ^a shows an rOe to the NH proton A′ of DACH(1). The energy-minimised model incorporating these constraints is shown in Figure 6(c).

pH Considerations

No buffers were used in these investigations, since buffer ions (e.g. phosphate) are potential ligands for Pt^{II}. The pH of each solution was regularly monitored during the course of each reaction. The pH of the glutathione and glutathione disulfide solutions was adjusted to 7 prior to addition of the platinum complex solution. Following addition of the platinum complex, the pH of the reaction mixture dropped within ca. 4 h to a value of between 4.5 and 5. The ^{1}H NMR spectra of products **A** and **B** show little change over the pH range 2.8−8.7, and therefore these species appear to be stable over a wide pH range.

Reactions of Oxaliplatin with GSH and GSSG

UV/Vis Measurements

The reaction between oxaliplatin (1 mL, 100 μM) and GSH (10 μL, 20 mM, pH 7) at 310 K gives rise to a new absorption band at 220 nm, which increases in intensity over a period of 48 h (Figure 8). The reaction between oxaliplatin and GSSG, which was monitored over 96 hours, is also slow and gives rise to a similar new absorption band. In comparison with the similar reactions of [Pt(*rac*-1,2-

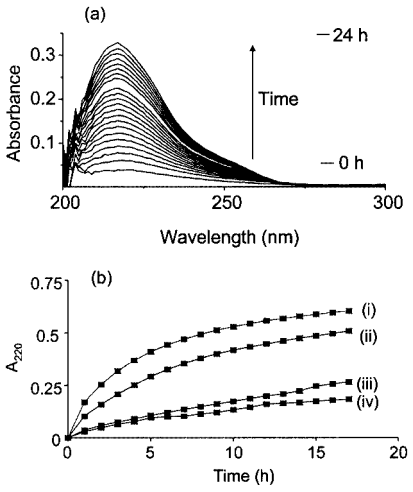


Figure 8. (a) The change in absorbance with time for reactions of oxaliplatin with two molar equivalents of GSH (initially at pH 7) in aqueous solution; measurements were taken at 310 K at regular hourly intervals for the first 24 hours of the reaction; (b) comparison of the changes in absorbance at 220 nm with time for reactions of [Pt(*rac*-1,2-DACH)Cl_2] or oxaliplatin with two molar equivalents of GSH or GSSG; labels for graphs: (i) [Pt(*rac*-1,2-DACH)Cl_2]/GSH, (ii) [Pt(*rac*-1,2-DACH)Cl_2]/GSSG, (iii) oxaliplatin/GSH and (iv) oxaliplatin/GSSG

DACH)Cl_2], those of oxaliplatin are approximately three times slower [Figure 8(b)].

HPLC Measurements

The HPLC chromatograms for reaction mixtures of oxaliplatin (2 mL, 100 μM) with two molar equivalents of GSH or GSSG (20 μL, 20 mM, pH 7, 310 K) using a 4−20% acetonitrile gradient over 30 min are shown in Figure 2(c) and (d). Peaks for two major products which elute at 14.8 min (**C**) and 15.5 min (**D**) appear in both chromatograms and increase in intensity over a period of 48 h, whilst the peaks corresponding to the reactants decrease in intensity. Small amounts of the starting materials were still present in the reaction mixtures after five days. Some minor peaks for unidentified products were also observed. For the GSH reactions, the relative areas of peaks **C** and **D** are similar, whereas for GSSG, peak **D** is more intense than peak **C**. A timecourse study (Figure S1 in the Supporting Information) showed that this preference for formation of product **D** was similar throughout the 0−24 h period that the reaction was studied.

ESI-MS Measurements

HPLC peaks **C** and **D** were collected for reactions between oxaliplatin and GSH or GSSG, and analysed by ESI-MS (Figure 9). Each peak corresponds to only one major product, with molecular ions at m/z = 615 (**C**) and m/z = 462 (**D**). Isotope modelling of the data showed that peak **C** was assignable to the molecular formula $[Pt_2C_{32}H_{58}N_{10}O_{12}S_2]^{2+}$ [the S-bridged dimer shown in Figure 6(a)] and peak **D** assignable to the formula $[Pt_2C_{22}H_{42}N_7O_6S]^{2+}$ [the glutathione-bridged macrochelate shown in Figure 6(b)]. These data are consistent with the

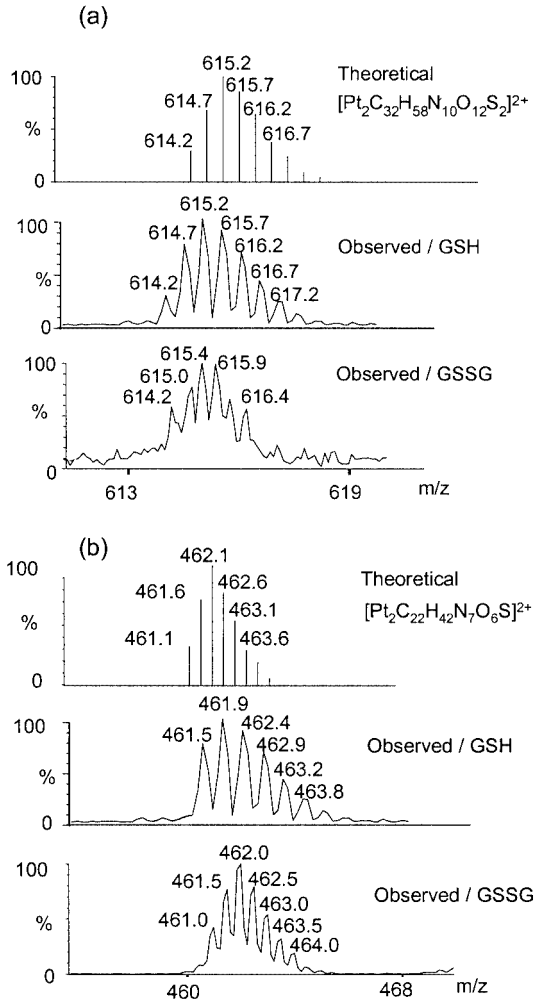


Figure 9. Positive ion ESI-MS spectra obtained from HPLC peaks (a) **C**, and (b) **D**, products from the reaction of oxaliplatin with two molar equivalents of GSH (310 K, initially at pH 7, six days), separated and collected using the elution conditions shown in Figure 2, and concentrated by lyophilisation; the observed and theoretical isotope patterns for the proposed structures are shown

products giving rise to peaks **C** and **D** from the oxaliplatin reactions being identical to those of peaks **A** and **B**, respectively, from the reactions of [Pt(1,2-DACH)Cl_2].

Discussion

Although cisplatin is an effective anticancer drug, a major clinical problem is the development of resistance.[4] Early on it was discovered that diaminocyclohexane (DACH) platinum(II) complexes are non-cross resistant with cisplatin and this has led to the clinical approval of one such DACH derivative, [Pt(1*R*,2*R*-DACH)(oxalate)] (oxaliplatin), for the treatment of metastatic colorectal cancer.[8,32] For [Pt(DACH)Cl_2] complexes containing the *trans*-DACH geometrical isomer, the *R*,*R* diastereomer is more active than *S*,*S*, and both are more active than the meso (*R*,*S*) adduct.[33] Since thiols such as the tripeptide glutathione (GSH), present in cells at concentrations of

0.5−10 mM,[16] can play a major role in cellular resistance mechanisms, there is a need to elucidate reactions between Pt-DACH anticancer complexes and glutathione.

In the only previous study (as far as we are aware) of such reactions, two products from the reaction of [Pt(1,2-DACH)Cl_2] and GSH were separated by HPLC.[34] They were found to be 1:1 (Pt:GSH) adducts, but were not further characterised. In this work we have studied reactions of both GSH and its oxidised form GSSG with both racemic (1*R*,2*R*/1*S*,2*S*) and enantiomerically pure [Pt(1*R*,2*R*-DACH)Cl_2], as well as the drug oxaliplatin. The time dependence of the reactions was followed by both UV/Vis spectroscopy and by HPLC. Remarkably, reactions of both GSH and GSSG gave rise to a similar broad band at ca. 220 nm (Figures 1 and 8), assignable to a thiolate-to-platinum charge-transfer band ($\varepsilon > 30{,}000\ M^{-1}cm^{-1}$). Reactions with GSSG were more than three times slower than with GSH. HPLC studies showed that reactions of [Pt(*rac*-1,2-DACH)Cl_2] with both GSH and GSSG gave rise to the same two major products [**A** and **B**, Figure 2(a) and (b)], which were identified by ESI-MS as 2(Pt):2(GSH) (**A**) and 2(Pt):1(GSH) (**B**) adducts.

Displacement of oxalate from oxaliplatin by GSH (or GSSG) leads to products **C** and **D**, which are identical to products **A** and **B** from reactions of the dichloro complex, from which the chloro ligands are displaced. Reactions of oxaliplatin with GSH and GSSG were much slower than those of [Pt(1,2-DACH)Cl_2]. This is not surprising in view of the presence of chelated oxalate in oxaliplatin.

Remarkably, therefore, GSSG undergoes redox reactions with platinum(II) complexes, leading to cleavage of the disulfide bond and formation of Pt^{II}-GSH adducts. Such redox chemistry of platinum(II) anticancer drugs has received relatively little previous attention. Lempers et al. have suggested that cleavage of GSSG by $[Pt(dien)Cl]^+$ involves H_2O as the reducing agent[16] and Fazlur-Rahman and Verkade have detected initial binding of GSSG to $[Pt(dien)]^{2+}$.[35] Ohta et al. have reported that *trans*-$[PtCl_2(NH_3)_2]$ can cleave disulfide bonds in human serum albumin,[36] and they also found that GSH reacts with cisplatin 17 times faster than with GSSG, and six times faster with the monoaqua adducts.[37] Further work is needed to elucidate the mechanism of such redox reactions.

The resolution of the reverse-phase HPLC chromatograms is very sensitive to the elution conditions (solvent gradient and temperature), but under certain conditions diastereomeric products were separable for reactions of the racemic complex. On the basis of ESI-MS and NMR spectroscopic data, product **A** can be identified as a thiolate-bridged dimer [Figure 6(a)] and product **B** as a novel dinuclear glutathione-bridged macrochelate [Figure 6(b)]. Evidence for the latter structure was provided by comparing 1D ^1H NMR, 2D [^1H, ^1H] TOCSY and NOESY data with those for products from similar reactions of the ethylenediamine complex [Pt(en)Cl_2], for which a comprehensive NMR study was possible with the use of ^{15}N-labelled en.[38] ^{15}N-DACH was not available for the present study.

In particular, similarities in the chemical shifts of the peptide ^1H NMR resonances and between the chemical shifts of the N*H* resonances of the diamine ligand are notable (Figures S3−S5 in the Supporting Information). A large doublet with a broadened base at $\delta = 6.33$ ppm, assignable to γ-Glu NH(1), is evidence for the presence of an unsymmetrical *bis* adduct. Differential couplings are consistent with torsion angles Cys−(Hβ^a−Cβ−Cα−Hα) = 90° and Cys−(Hβ^b−Cβ−Cα−Hα) = 30°; no signal was detected for Cys−N*H*, consistent with formation of a Cys−N(S) chelate ring. The macrochelate **B** contains a five-membered N,S chelate ring formed by the deprotonated amide N and S of the cysteine residue of GSH, and a nine-membered macrochelate ring formed by coordination of the γ-Glu α-amino group to the second Pt(DACH) unit.

Although the S-bridged dimer **A** is likely to be a stable end product, the macrochelate **B** is expected to be more reactive. Since we found that **B** is still formed even in the presence of a large excess of GSH (as would be the case inside cells), further studies of this species may well have physiological significance. Protonation of the Cys−N would produce a reactive coordination site, and the pH on the surface of DNA may be as low as 5;[39] however, complex **B** appeared to be stable down to pH 2.8.

Conclusions

Previous studies of the mechanism of action of platinum anticancer drugs have mostly been centred on the nature of hydrolysis reactions that activate chloro complexes, and reactions with DNA bases that lead to structural changes such as DNA bending. Whilst it is well recognised that platinum(IV) anticancer drugs are readily reduced in physiological media giving active platinum(II) products,[40] and that Pt^{II} can react with amides and imides to form mixed-valence "platinum blues",[41] little attention has been given to redox reactions of platinum(II) drugs with disulfides. We have shown here that reactions of the drug oxaliplatin with glutathione disulfide are only slightly slower than analogous reactions with the reduced form of glutathione. Remarkably, reactions of oxaliplatin with glutathione and glutathione disulfide lead to the same major products, both platinum(II) adducts of glutathione. Clearly, oxaliplatin can induce reductive cleavage of the disulfide bond of glutathione disulfide. Since platinum is still in oxidation state +2 in the products, the electrons must be supplied by another component of the reaction, probably water. The formation of the unusual macrochelate **B**, even in the presence of excess glutathione, could potentially be of biological importance.

Experimental Section

Materials: Potassium oxalate, *rac*-1,2-DACH and (1*R*,2*R*)-DACH were purchased from Aldrich. Potassium tetrachloroplatinate(II) was provided by Johnson Matthey. Enantomerically pure [Pt(1*R*,2*R*-DACH)Cl_2], [Pt(*rac*-1,2-DACH)Cl_2] and oxaliplatin

were prepared using previously described procedures.[42−45] Glutathione (GSH) was purchased from Acros and glutathione disulfide (GSSG) from Sigma.

Instrumental Methods: High pressure liquid chromatography (HPLC) was carried out on a Hewlett Packard 1100 system with UV detection. For all analytical separations, a RP C18 column (250 × 4.6 mm, 100 Å, 5 μM, Hichrom) with UV detection at 220 nm, eluting with 0−50% acetonitrile gradients over varying time intervals with 0.1% trifluoroacetic acid as an ion-pairing agent. ESI-MS positive ion electrospray mass spectrometry (ESI-MS) was performed using a Platform-II mass spectrometer (Micromass, Manchester, UK). A Corning 240 pH meter equipped with an Aldrich microcombination electrode standardised with Aldrich buffers at pH 4, 7 and 10 was used to make pH measurements. Electronic absorption spectra were recorded over a range of 800 to 200 nm at 310 K on a Perkin−Elmer Lambda-16 spectrometer using a cell with a path length of 1 cm. ICP-AES determination of platinum concentrations was carried out using a Thermo Jarrell Ash *IRIS* ICP-AES, calibrated with 0−100 ppm standards purchased from BDH.

NMR spectroscopic data were acquired using a Varian ^{UNITY}*INOVA* 600 NMR spectrometer equipped with a z-field gradient triple resonance NMR probe and operating at a ^{1}H resonance frequency of 599.841 MHz. A sample of the 2:1 adduct between [Pt(1*R*,2*R*-DACH)Cl$_2$] and GSH (1.3 mM, 90% H_2O/10% D_2O, pH = 4, separated by HPLC) was used for the acquisition of all NMR spectroscopic data. 1D ^{1}H NMR spectroscopic data sets were typically acquired under the following conditions: frequency width 7 kHz, acquisition time 2.34 s, number of data points 32 *k*, relaxation delay 2 s, number of transients 128, pulse angle 90°. Homonuclear ^{1}H 2D NOESY (τ_m = 150 and 500 ms), TOCSY (τ_m = 70 ms), DQFCOSY and ROESY (τ_m = 120 ms) NMR spectroscopic data sets were typically acquired over a 5 kHz frequency width in both ω2 and ω1 for which 32 transients were acquired into 2 k complex data points (acquisition time 205 ms) for each of 2 × 400 hypercomplex t_1 increments. For all data accumulation, solvent suppression was achieved using either WET[46] or excitation sculpting.[47] The sample temperature was maintained at 298 K throughout data acquisition. Data were converted (Xwin-NMR version 2.0, Bruker, U.K. Ltd.) and processed using suitable apodization functions. Referencing of all data was made internally to the methyl singlet of TSP at δ = 0 ppm. A model for [{Pt(1*R*,2*R*-DACH)}$_2$(μ$_2$-*S*G)] was constructed on the basis of the structure calculated for [Pt(en)$_2$(μ$_2$-*S*G)][38] using Sybyl (version 6.3, Tripos Inc.). The presence of an rOe in the NMR spectroscopic data was used to define upper (3.5 Å) and lower (2.5 Å) distance bounds between associated protons in the molecule. Distance range force constants, k_{NOE}, were set to 20 kcal·mol^{-1} Å^{-2} for each distance restraint. A single torsion angle restraint was also incorporated (γ-Glu−(Hα−Cα−Cβ−Hβ^a); θ_{tor} = 90°, k_{tor} = 0.01 kcal·mol^{-1} deg^{-2}). Energy minimization of the model was carried out with repeated conjugate gradient steps until the structure was stable.

Reactions of [Pt(1,2-DACH)Cl$_2$] and Oxaliplatin with Glutathione (GSH) or Glutathione Disulfide (GSSG): The pH of GSH or GSSG solutions (20 mM) was adjusted to 7 with NaOH. [Pt(1,2-DACH)Cl$_2$] or oxaliplatin (100 μM, 2 mL) was added to an aliquot of GSH or GSSG (20 μL, 2 mol equiv.), and the reaction mixture was incubated at 310 K for 24 h ([Pt(1,2-DACH)Cl$_2$]) or 48 h (oxaliplatin). The pH of the reaction was monitored at regular intervals. Analytical separation of the products was carried out by HPLC methods as described above.

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