Inorg. Chem. **2003**, *42*, 5498–5506

Reaction of Polynuclear Platinum Antitumor Compounds with Reduced Glutathione Studied by Multinuclear (^{1}H, ^{1}H–^{15}N Gradient Heteronuclear Single-Quantum Coherence, and ^{195}Pt) NMR Spectroscopy

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Received January 31, 2003

A possible explanation for the low bioavailability of platinum antitumor compounds is their high reactivity with the sulfur-containing tripeptide glutathione (GSH; deprotonated GSH = SG). GSH is located in the intracellular matrix of the cell with a normal concentration of 5–10 mM. In vivo, only a small fraction of the administered drug will migrate into the cell, resulting in relatively high concentrations of GSH compared to that of the drug. The products of the reactions of [{*trans*-PtCl(NH$_3$)$_2$}$_2$-μ-{*trans*-Pt(NH$_3$)$_2$(NH$_2$(CH$_2$)$_6$NH$_2$)$_2$}](NO$_3$)$_4$ (BBR3464; 1,0,1/*t*,*t*,*t*, n = 6), [{*trans*-PtCl(NH$_3$)$_2$}$_2$-μ-(H$_2$N(CH$_2$)$_6$NH$_2$)](NO$_3$)$_2$ (BBR3005; 1,1/*t*,*t*, n = 6), [{*trans*-PtCl(NH$_3$)$_2$}$_2$-μ-(H$_2$N(CH$_2$)$_3$NH$_2$-(CH$_2$)$_4$NH$_2$)]Cl$_3$ (BBR3571; 1,1/*t*,*t*-spermidine, n = 3, 4), and *trans*-[PtCl$_2$(NH$_3$)$_2$] (*t*-DDP) with reduced GSH in phosphate-buffered saline (pH 7.35) have been characterized by ^{1}H, ^{195}Pt, and ^{1}H^{-15}N gradient heteronuclear single-quantum coherence NMR spectroscopy and high-performance liquid chromatography (HPLC) coupled with electrospray ionization time-of-flight mass spectrometry to determine likely metabolites of the complexes with GSH. Chemical shifts (NMR) and retention times (HPLC) established via analysis of the *t*-DDP profile served as a fingerprint to compare results obtained for the products afforded by the degradation of the polynuclear compounds by GSH. Identical kinetic profiles and chemical shifts between the metabolites and the *t*-DDP/GSH products allowed identification of the final product for the 1:2 Pt:GSH reaction as a dinuclear species [{*trans*-Pt(SG)(NH$_3$)$_2$}$_2$-μ-SG], in which glutathione bridges the two platinum centers via only the sulfur atom.

Introduction

Cisplatin (*cis*-[PtCl$_2$(NH$_3$)$_2$]) is an effective anticancer agent with cytotoxicity primarily mediated through interactions with DNA. Bifunctional binding to DNA of the *cis*-[Pt(NH$_3$)$_2$]$^{2+}$ unit to two adjacent N7 atoms of guanine with the formation of intrastrand and interstrand cross-links causes an inhibition of DNA replication.[1−4] The wider clinical application has been hampered by resistance and associated toxic side effects, in particular dose-limiting nephrotoxicity.[5] From its serendipitous discovery to the clinic, cisplatin has brought platinum therapy to the forefront of cancer treatment.

Our efforts in metal complex chemistry have focused on structurally novel platinum complexes. The research is guided by the premise that these agents would maintain a mode of DNA adduct formation different from that of cisplatin, and exhibit complementary activity within cisplatin-resistant cell lines.[6]

The dinuclear/trinuclear complexes, as depicted in Chart 1, represent a novel class of platinum-based chemotherapeutic agents that contain two or three platinum centers linked by an alkanediamine chain.[6,7] The first of these "nonclassical" platinum complexes to enter clinical trials, [{*trans*-PtCl(NH$_3$)$_2$}$_2$-μ-{*trans*-Pt(NH$_3$)$_2$(NH$_2$(CH$_2$)$_6$NH$_2$)$_2$}](NO$_3$)$_4$ (1,0,1/*t*,*t*,*t*, n = 6) (**2**), is more potent than its dinuclear analogue [{*trans*-PtCl(NH$_3$)$_2$}$_2$-μ-(H$_2$N(CH$_2$)$_6$NH$_2$)](NO$_3$)$_2$ (1,1/*t*,*t*, n = 6) (**1**) and significantly more so than its predecessor cisplatin.[8] Clinically, an effective dose significantly less than

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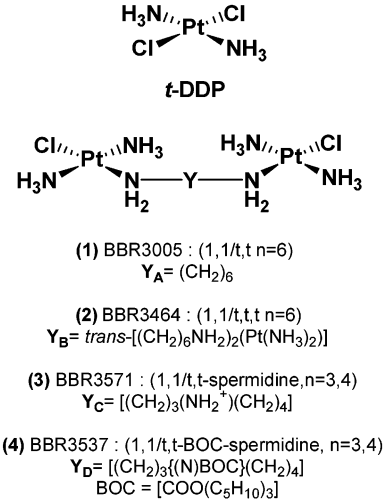
10.1021/ic030045b CCC: $25.00 © 2003 American Chemical Society
Published on Web 08/07/2003

Chart 1. Mono- and Polynuclear Platinum Drugs Used in This Study[a]

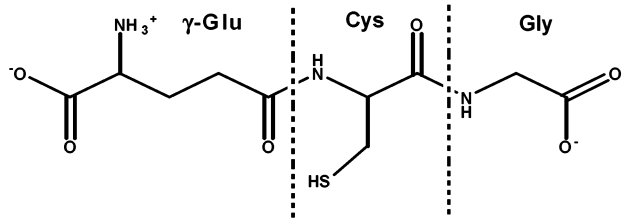


[a] Charges omitted for clarity.

that of cisplatin is used for **2** (1 mg/m^2 versus 60−120 mg/m^2, respectively).[9,10] The increased activity of **2** has been attributed to a combination of enhanced cellular uptake and enhanced target (DNA) affinity.[11] **2** has undergone phase II clinical trials for the treatment of small-cell lung, non-small-cell lung, ovarian, and gastric cancer.[12,13]

Although attack on DNA is the accepted cause for antitumor activity,[1−4] many platinum complexes have the potential to interact with other biomolecules, especially sulfur-containing compounds. Some examples of sulfur-containing substrates present in vivo include the amino acids cysteine and methionine, the tripeptide glutathione, and proteins such as metallothionein and human serum albumin. These molecules are generally considered to be responsible for the metabolic (nontarget) interactions of cisplatin.[14,15] Glutathione (GSH) (Chart 2) has been chosen for investigation in this study because of its role as a determinant of cellular sensitivity to a wide variety of drugs and cytotoxic agents. With normal intracellular concentrations of GSH ranging from 5 to 10 mM,[16] the direct coordination of GSH to platinum-containing drugs is highly probable, given the propensity of platinum(II) to form a stable bond with the soft base sulfur.[17] Confirmation of Pt−S protein coordination is provided by the observed irreversible inhibition of the vital redox enzymes trypanothione reductase[18] and human thioredoxin reductase.[19] Sadler et al. have also reported the coordination of platinum to the sulfur of free cysteine and methionine residues on the surface of the plasma protein human serum albumin.[20]

Chart 2. Chemical Structure of GSH



Investigations of the reaction of *cis*platin and *trans*platin with reduced GSH in human red blood cells concluded that the *trans* isomer reacted with GSH to form a simple mononuclear complex, *trans*-[Pt$(NH_3)_2(SG)_2$].[21] In contrast, NH_3 ligands were eventually liberated from *cis*-[$PtCl_2(NH_3)_2$], which resulted in a high molecular weight bridged species with a 1:2 Pt:GSH stoichiometry.[22,23] The tendency of coordinated Pt−SG species to form bridged dinuclear Pt_2-μ-SG species has also been shown for [PtCl(dien)]$^+$, where the presence of only one leaving group in the monofunctional compound facilitated kinetic studies.[24] The bridged complex is thought to be a consequence of the increased nucleophilic nature of the Pt-bound sulfur, Pt−SG (or Pt−S−R in general), upon proton displacement by Pt. Interestingly, sulfur substitution is considered to occur through direct attack on the chloride complex, prior to aquation. The analogous reaction with *cis*-DDP would give a [$(Pt(NH_3)_2)$-μ-$(SG)_2$] core. Crystallographic evidence for this structure has been provided for bridging *N*-acetylcysteine in the compound [Pt_2(μ-*N*-acetyl-L-cysteine-*S*)$_2$(bpy)$_2$].[25] Likewise, a bridged species has been identified as the major product from the reaction of [$PtCl_2$(en)] with GSH.[26] Minor products include an unusual bridged form with one glutathione bound per two Pt(en) moieties and where chelation occurs through a combination of the sulfur and peptide nitrogen.

Both clinical and preclinical studies have shown that cells with an elevated level of GSH (>10 mM) may be resistant

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to varying degrees to cisplatin.[27] Indeed, the development of the phase II clinical agent *cis*-[$PtCl_2(NH_3)$(2-picoline)] (ZDO473/AMD473) was predicated on its reduced reactivity with GSH, resulting in potential enhanced activity in those cell lines where elevated GSH contributes to resistance.[28−32] Glutathione may also interfere with or reverse Pt−DNA binding, resulting in loss of adduct structure.[33] GSH interactions may alter the toxicity or cause a lack in efficacy of administered drug by quenching potential DNA−Pt monoadducts before the rearrangement into toxic bifunctional adducts may occur.[27,34,35]

Highly active polynuclear complexes also contain Pt−Cl bonds, and it is therefore of interest to examine their reactions with sulfur-containing biomolecules, to give a complete understanding of the cellular pharmacology of the drug. In this paper we report a study of the reactions of *trans*-[$PtCl_2(NH_3)_2$] (*t*-DDP) and several novel polynuclear platinum compounds (see Chart 1) with glutathione under physiological conditions (pH 7.35, 37.5 °C). Extensive studies on DNA binding have clearly indicated the effect the central Pt(amine)$_4$ unit exerts on the structure and kinetics of formation of Pt−DNA adducts of related dinuclear and trinuclear complexes. These differences may also be manifested in metabolic reactions. Interaction of polynuclear drugs with the nucleophilic sulfur of the cysteine residue could potentially result in the liberation of the terminal secondary amine of the alkanediamine linker. Therefore, drug degradation via glutathione interactions could result in decreased bioavailability, as well as increased amounts of toxic metabolites in the host system. During the course of this work, a brief report on the reaction of GSH with the dinuclear platinum drugs developed in our laboratory appeared.[36]

To present the experimental data obtained in a succinct manner, the results are ordered so that the reaction of *t*-DDP with GSH will be first discussed as a model for comparison with the results obtained from the reactions involving the polynuclear species.

Experimental Section

Sample Preparation. 1, **2**, [{*trans*-$PtCl(NH_3)_2$}$_2$-μ-($H_2N(CH_2)_3NH_2(CH_2)_4NH_2$)]$Cl_3$ (**3**), and *t*-DDP (normal and ^{15}N-labeled) were all prepared by previously reported procedures.[7,37,38] Glutathione (99% reduced) was purchased from Aldrich Sigma.

A stock solution of both deuterated (DPBS) and normal (PBS) phosphate-buffered saline ([phosphate] = 150 mM, pH 7.35 at 37° C, [NaCl] = 120 mM, [KCl] = 2.7 mM) was prepared for all reaction solutions to mimic physiological conditions. Previous experiments utilizing PBS at lower concentrations of phosphate (50, 75, and 100 mM) did not possess the buffering capacity required to maintain a pH between the values of 7.2 and 7.4. The pH of all solutions prepared in this study was measured at both pre- and postreaction times with values never below 7.19.

A final ratio of 2:1 GSH:Pt−Cl was used for reactions of dinuclear and trinuclear compounds. The central platinum of **2** is considered to be inert and was not included in any ratio calculations. Reactions involving *t*-DDP were conducted at a 1:2 Pt:GSH ratio except when a ratio of 2:3 Pt:GSH was utilized to prevent the reaction from reaching completion, allowing the determination of intermediates.

Prior to the analysis of any reaction, each sample was vortexed for 5 min and then sonicated for an additional 10 min to ensure homogeneity. Transplatin is sparingly soluble in water, yet the product afforded by the coordination of sulfur to platinum is soluble. Therefore, each reaction started as a pale yellow suspension and over a period of ∼1.5 h would progressively become a darker shade of yellow and then slowly revert back to clear. Only in reactions where the Pt:GSH ratio was 2:1, as opposed to 1:2 Pt:GSH, would a pale yellow precipitate form.

High-Performance Liquid Chromatography (HPLC). *t*-DDP/GSH (1:2) reactions were monitored using a Jasco MD 600 HPLC instrument with a Phenomenex, Jupiter C_{18}, 5u, 250 × 4.6 mm, 300 Å, column and a 50 μL sample injection loop. Chromatograms were acquired at λ 255 nm for a total of 50 min. A ternary eluent gradient was used in this study at a flow rate of 1 mL/min. Eluent A was comprised of a 95:5 mixture of H_2O/ACN, eluent B a 80:20 mixture, and eluent C a 50:50 mixture. All three eluents contained 2.5 g/L octanesulfonic acid and were titrated to a pH of 2.7 with concentrated phosphoric acid. The gradient method used for this analysis was as follows: 100% A to 100% B in 10 min; 100% B to 100% C in 20 min; 100% C for 20 min; back to 100% A in 5 min; reequilibration at 100% A for an additional 5 min.

NMR Spectroscopy. ^{1}H NMR and ^{195}Pt NMR spectra were recorded on a Varian Mercury series 300 MHz NMR spectrometer using a 5 mm probe for ^{1}H and a 10 mm broad-band probe for ^{195}Pt. ^{1}H spectra were referenced to sodium 3-(trimethylsilyl)-propionate (TSP) at δ = 0 ppm, and ^{195}Pt spectra were referenced to $Na_2[PtCl_6]$. The scanning frequency for ^{195}Pt nuclei was set at 64.32 MHz.

^{1}H−^{15}N Gradient Heteronuclear Single-Quantum Coherence (HSQC). HSQC NMR spectra were recorded on a Varian Inova 400 MHz NMR spectrometer with an indirect-detection 5 mm probe. Samples were dissolved in 92:8 H_2O/D_2O. The pulse sequence used was obtained from work previously published by Kay et al.[39] ^{15}N spectra were referenced to $^{15}NH_4NO_3$ at δ = 0 ppm. Decoupling was obtained by use of the WALTZ[40] sequence using a 1 kHz radio frequency field. Water suppression was

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achieved by using the gradient pulses. A total of 1024 points were acquired in the ^{1}H dimension with a sweep width of 2000 Hz for each of 128 complex points and in the ^{15}N dimension with a sweep width of 4823 Hz. Eight transients were used.

Electrospray Ionization Time-of-Flight Mass Spectrometry (ESITOFMS). Mass spectrometric analysis was carried out on a Micromass (Micromass UK Ltd.) LCT time-of-flight mass spectrometer fitted with a dual electrospray source. All spectra were internally calibrated using a 10 μM solution of poly(ethylene glycol) (PEG) with an average molecular weight of 1000.[41−43] By the use of an internal calibrant, PEG, the *m*/*z* signal acquired at 1376.25 was determined to have a mass accuracy of 5 ppm.

Calculation of the Rate of Formation of [{*trans*-Pt(SG)-$(NH_3)_2$}$_2$-μ-SG] from *trans*-[Pt(SG)$_2$$(NH_3)_2$]. HPLC could also be used to obtain the approximate rate at which the intermediate reacted to form the bridged final product. At 4−5 h, the concentrations of both species are approximately equal; the rate of the subsequent change in area for the two peaks assigned as *trans*-[Pt(SG)$_2$$(NH_3)_2$] (21 min) and [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (32 min) were recorded until only 0.04% of the *trans*-[Pt(SG)$_2$$(NH_3)_2$] peak remained at 8 h. The data points obtained from HPLC measurements were fit to a smooth curve generated by SCIENTIST (MicroMath Scientific Software). These were characterized by the property that the rate of product formation is proportional to the amount of reactant. The rate of change from *trans*-[Pt(SG)$_2$$(NH_3)_2$] to the bridged [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG], using the equation $d[A]/dt = -k[A]$, was calculated to have an approximate k value of 0.4033 s^{-1} (SD = 0.05).

Results

Reaction of *trans*-[PtCl$_2$$(NH_3)_2$] with GSH. Profile and Characterization of Products. (a) ^{195}Pt NMR Spectroscopy. The reaction of *trans*-[PtCl$_2$$(NH_3)_2$] with GSH was monitored using ^{195}Pt NMR spectroscopy to determine the reaction profile over a period of time. A spectrum acquired at t = 12 h of a 1:2 *t*-DDP:GSH molar ratio reaction exhibited only one peak at −3186 ppm. The chemical shift value obtained is consistent with coordination of the deprotonated Cys thiol of GSH to the platinum atom.[21−23] Using a stoichiometry of 2:3 *t*-DDP:GSH, two peaks [δ −3186 ppm (**VI**) and δ −3226 ppm (**V**)] were resolved, which remained relatively unchanged for a time period of 7.5 h. An additional equivalent of GSH (2:4 *t*-DDP:GSH) was added to the solution to drive the reaction to its major product. After 30 min, the completion of the reaction was indicated by the appearance of only one peak resolved at δ −3186 ppm (Figure 2A).

(b) Chromatography. HPLC (spectra not shown) was vital as a technique in the isolation of the final product of a 1:2 *t*-DDP:GSH reaction. Multiple intermediates were detected up to t = 4 h, after which two peaks of equal intensity, provisionally assigned by ^{195}Pt NMR as the bissulfur and bridged species, were observed at retention times (RTs) of 21 and 32 min, respectively. Over the next 8 h, there was a decrease in the area of the first peak (RT = 21 min) with a correlated increase in area of the second peak, until only the second peak at RT = 32 min remained (99.6% total area).

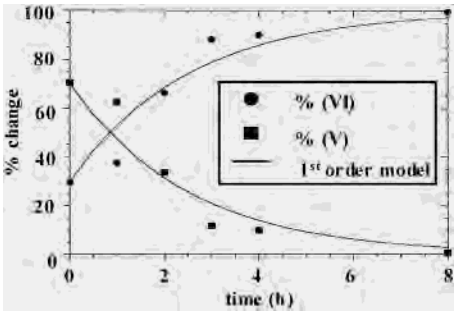

Figure 1. SCIENTIST plot exhibiting the rate of formation of **VI** from **V**. The smooth curved line represents the first-order model generated by the SCIENTIST program. The k value was calculated to be 0.4033 s^{-1} with a standard deviation 0.048 s^{-1}.

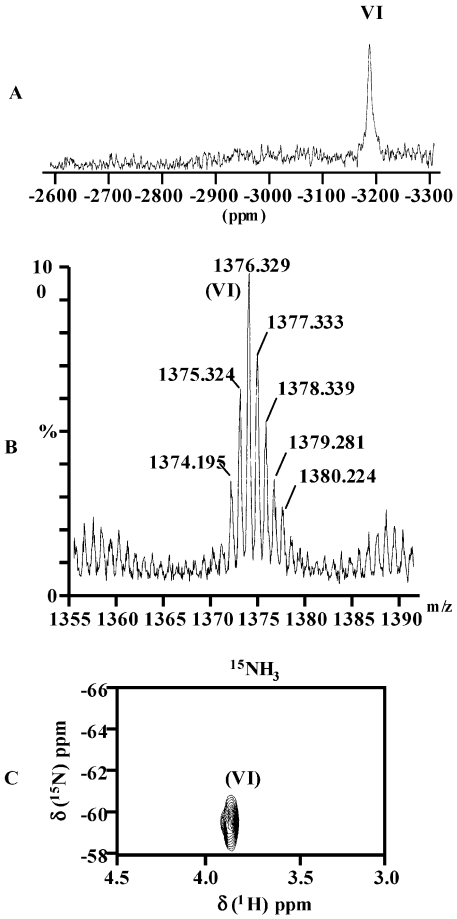

Figure 2. Spectra of the final product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (**VI**) afforded from a 4:1 GSH:*t*-DDP reaction in 150 mM DPBS, pH 7.35 at 37 °C: (A) ^{195}Pt NMR spectrum; (B) ESITOF mass spectrum; (C) ^{1}H−^{15}N HSQC spectrum.

(c) ESITOFMS. An aliquot of the final product (32 min) of the 1:2 *t*-DDP:GSH reaction collected via HPLC was then analyzed using ESITOFMS to determine the exact mass. Four major *m*/*z* signals were acquired from this sample. Reduced GSH and oxidized GSSG signals were observed at *m*/*z* values of 308.12 and 613.17, respectively. Two other signals at *m*/*z* = 1376.25 and 687.91 are consistent with the +1 and +2 charged states of the bridged complex [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG]. No other signals assignable to *trans*-

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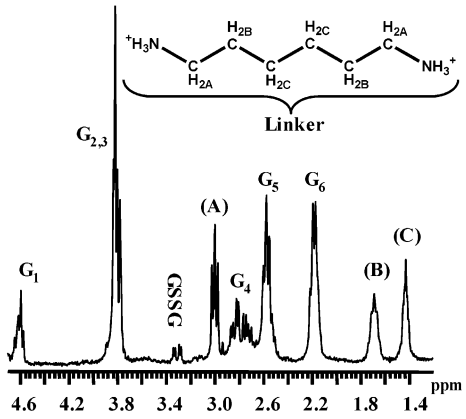

Figure 3. ^{1}H NMR spectra acquired of a 1:4 **2**:GSH reaction in 150 mM DPBS, pH 7.35, 37 °C, at $t = 12$ h, where G_1 = Cys α-CH_2, $G_{2,3}$ = Gly α-CH_2 and Glu α-CH_2, respectively, G_4 = Cys β-CH_2, G_5 = Glu γ-CH_2, and G_6 = Glu β-CH_2. The spectra were primarily used for the confirmation of drug degradation into a free hexanediamine unit and two mononuclear platinum units by the observance of the triplet at 3.0 ppm.

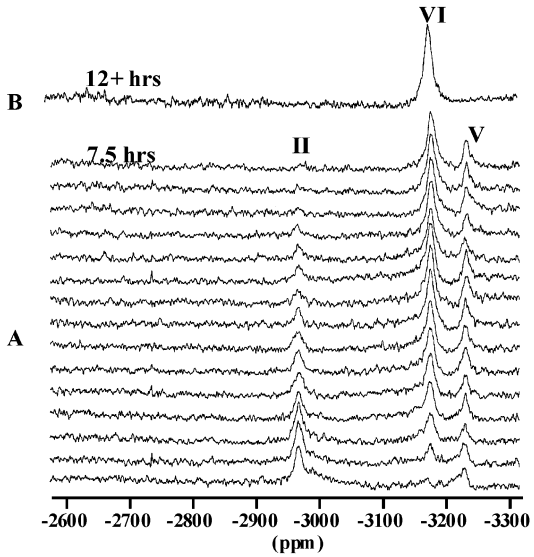

Figure 4. (A) Reaction of **1** with GSH (ratio 1:4) at 37 °C in DPBS (pH 7.4) as monitored by ^{195}Pt NMR from $t = 0.5$ h to $t = 7.5$ h. (B) Spectrum of the final product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (**VI**) of the 1:4 **1**:GSH reaction at $t = 12+$ h.

$[Pt(NH_3)_2(SG)_2]$ were observed. Thus, the bridged species has a ^{195}Pt NMR peak at −3186 ppm and HPLC retention time of 32 min under the buffer conditions used here. No other signals were observed for the +1 or +2 state of the bissulfur complex, thus an indication that the peak observed previously at 32 min by HPLC was solely the bridged [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (see Figure 2B).

An aliquot of the 1:2 *t*-DDP:GSH reaction was collected after only 60 min of reaction time. ESITOFMS (spectrum not shown) was conducted on this sample and indicated that GSH, GSSG, *trans*-$[Pt(SG)_2(NH_3)_2]$, and [{*trans*-Pt(SG)-$(NH_3)_2$}$_2$-μ-SG] are all present in solution at this time period.

(d) ^{1}H−^{15}N HSQC. ^{15}N-labeled *trans*-$[PtCl_2(NH_3)_2]$ reacted with GSH at a 2:3 *t*-DDP:GSH reaction ratio was analyzed at $t = 5$ h using ^{15}N−^{1}H HSQC. Two peaks of equal intensity with δ(^{1}H/^{15}N) shifts for the Pt−$(NH_3)_2$ moiety were observed at 3.52/−62.7 and 3.83/−59.1 ppm. Additional GSH was then added to the reaction for a ratio of 2:4 *t*-DDP:GSH and the reaction allowed to proceed for an additional 2 h. A second spectrum, Figure 2C, was then acquired at $t = 8$ h, in which only one peak ([{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG]) with a ^{1}H/^{15}N chemical shift of 3.83/−59.1 ppm was observed. It was then possible to conclude from retention times obtained from HPLC and chemical shifts from NMR spectra that the intermediate *trans*-$[Pt(SG)_2(NH_3)_2]$ and a final product, [{*trans*-Pt(SG)-$(NH_3)_2$}$_2$-μ-SG], were now successfully identified.

Reactions of 1 (1,1/*t*,*t*) with GSH. (a) ^{1}H NMR Resonance Assignment. A 1:4 **1**:GSH reaction between GSH and the dinuclear compound [{*trans*-PtCl$(NH_3)_2$}$_2$-μ-($H_2N(CH_2)_6$-NH_2)]$(NO_3)_2$ (1,1/*t*,*t*, $n = 6$; **1**) was followed by ^{1}H NMR spectroscopy (see Figure 3). The triplet at 2.79 ppm corresponding to the (CH_{2A}) protons of the linked 1,6-hexanediamine suffers a downfield shift to 3.0 ppm within 0.5 h. This is consistent with the liberation of the terminal amine and was confirmed by comparison with the control spectrum of free 1,6-hexanediamine. This was a simple and direct way to determine whether degradation of the drugs upon coordination of GSH was occurring. Due to excessive overlap of the signals induced by GSH and the drug, ^{1}H NMR spectroscopy was not utilized in this study, nor in the study of **2**, as a method to measure reaction progression.

(b) ^{195}Pt NMR Spectroscopy. The ^{195}Pt NMR spectrum of free **1** gave a single peak with a chemical shift of −2397 ppm.[44] An initial spectrum, as seen in Figure 4A, was then acquired of a 1:4 **1**:GSH reaction with three new peaks observed at −2987, −3186, and −3229 ppm within 30 min. Over 7 h, a continuous decay of the intermediate peak at −2987 ppm was seen with an associated increase of the peak at −3186 ppm. A final spectrum at $t = 12+$ h (Figure 4B) exhibited only one peak at −3186 ppm. The peak at −2987 ppm is assigned to formation of the dinuclear intermediate [{*trans*-Pt(GS)$(NH_3)_2$}$_2$-μ-($NH_2(CH_2)_6NH_2$)$_2$]. The other two peaks correspond to the mononuclear *trans*-$[Pt(SG)_2(NH_3)_2]$ (−3229 ppm) and bridged [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (−3186 ppm) as previously assigned.

(c) ^{1}H−^{15}N HSQC NMR. As can be seen in Figure 5, HSQC spectroscopy proved to be useful in the identification of the reaction intermediate [{*trans*-Pt(GS)$(NH_3)_2$}$_2$-μ-($NH_2(CH_2)_6NH_2$)]. Two peaks were observed at 5.04/−46.0 ppm, δ(^{1}H/$^{15}NH_2$), and 3.87/−63.4 ppm, δ(^{1}H/$^{15}NH_3$), for unreacted **1**.[7] Upon the initiation of a 1:4 **1**:GSH reaction, the peak (5.04/−46.0 ppm) of the terminal Pt−$^{15}NH_2$ of the linker for **1** was no longer observed. Due to the large separation of the chemical shifts between the $^{15}NH_2$ and $^{15}NH_3$ regions of the spectrum, however, ^{1}H−^{15}N HSQC allowed the identification of the intermediate species via the associated chemical shifts of $^{15}NH_3$. As observed in Figure 5, three peaks were observed in the $^{15}NH_3$ region of the spectrum at $t = 4$ h. The peak at 3.77/−62.5 ppm decayed over time to a complete loss of signal at exactly 7 h.

A correlation could then be made by comparison with the ^{195}Pt NMR spectra, as the profile of signal intensity is

(44) Qu, Y.; Farrell, N. *Inorg. Chem.* **1992**, *31*, 930−932.

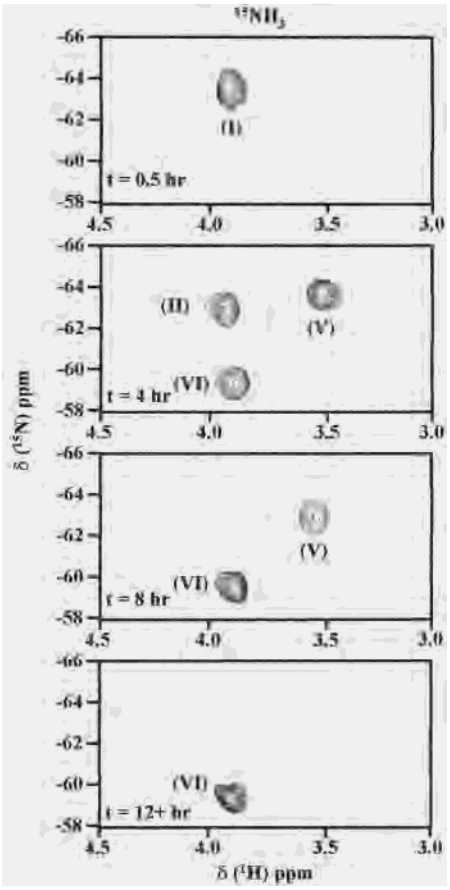

Figure 5. $^1H-^{15}N$ HSQC spectrum of the reaction of **1** with GSH (ratio 1:4) at 37 °C in DPBS (pH 7.4) from $t = 0.5$ h to $t = 12+$ h.

consistent. As was depicted in Figure 4, exactly 7 h passed before a signal could no longer be resolved via Pt NMR for the intermediate [{*trans*-Pt(GS)$(NH_3)_2$}$_2$-μ-$(NH_2(CH_2)_6NH_2)$], and the peaks at 3.77/−62.5 ppm are assigned to this species. The two remaining peaks were then assigned as the mononuclear *trans*-[$Pt(SG)_2(NH_3)_2$] (3.53/−62.8 ppm) and bridged [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (3.85/−59.1 ppm). A transition of the mononuclear *trans*-[$Pt(SG)_2(NH_3)_2$] to the final product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] was also observed up to $t = 8$ h.

Reactions of 2 (1,0,1/*t*,*t*,*t*) with GSH. (a) ^{1}H NMR Resonance Assignment. On the basis of the mononuclear and dinuclear experiments, the 4:1 reactions of GSH with **2** may be readily interpreted. In the ^{1}H NMR spectrum (spectrum not shown) the appearance of a new peak at 3.00 ppm is indicative of the production of the free amine; however, unlike the dinuclear case, the signal at 2.7 ppm does not disappear completely. For the trinuclear compound cleavage of the Pt−NH_2 terminal bonds (i.e., *trans* to Cl) would produce the linker *trans*-[$Pt(NH_3)_2(H_2N(CH_2)_6NH_3^+)_2$].

(b) ^{195}Pt NMR Spectroscopy. The ^{195}Pt NMR spectrum of free **2** (figure not shown) showed two peaks at −2417 and −2648 ppm.[45] Upon the 1:4 **2**:GSH reaction, the peak acquired at −2417 ppm (assigned as a PtN_3Cl coordination sphere) was no longer resolvable after 0.5 h.

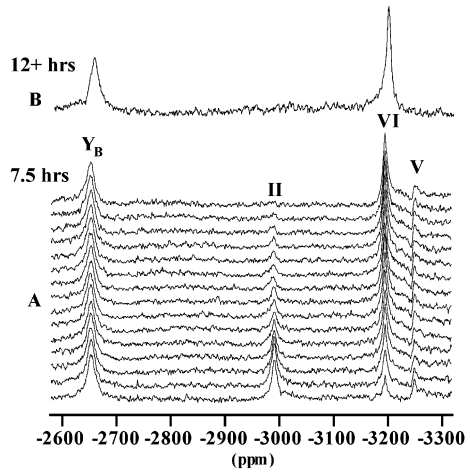

Figure 6. (A) Reaction of **2** with GSH (ratio 1:4) at 37 °C in DPBS (pH 7.4) as monitored by ^{195}Pt NMR from $t = 0.5$ h to $t = 7.5$ h. (B) Spectrum of the final product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (**VI**) of the 1:4 **2**:GSH reaction at $t = 12+$ h. Note that the signal for the central platinum of **2** remains constant throughout, an indication that the subunit $\mathbf{Y_B}$ is an inert species.

The upfield peak at −2648 ppm, assigned to the central platinum of **2**, remained constant throughout the reaction, indicating that the central platinum did not react with GSH. As shown in Figures 5 and 6, there are no major differences (besides the signal at −2648 ppm) between the spectra acquired for the 1:4 reaction of **1** and **2**. Each shows a loss of signal for the intermediate [{*trans*-Pt(GS)$(NH_3)_2$}$_2$-μ-$(NH_2(CH_2)_6NH_2)_2$] by 7 h, and by 12+ h reveals only the final bridged product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] at −3185 ppm.

(c) ^{1}H−^{15}N HSQC NMR. The control spectrum acquired for **2** revealed a total of four peaks, $\delta(^1H/^{15}N)$, two peaks at 5.06/−47.1 and 4.61/−42.9 ppm for $^{15}NH_2$ and two peaks at 3.87/−63.4 and 4.08/−62.1 ppm for $^{15}NH_3$ coordinated to the terminal platinum and central platinum atoms, respectively.[46] As can be seen in Figure 7, the ^{15}N signals for the nitrogen coordinated to the central platinum remain constant over 12+ h. A loss of the terminal $^{15}NH_2$ signal occurs within the first 0.5 h. No corresponding peak was observed for possible Pt−SG coordination (**II**), possibly because of its close proximity to the water peak.[46] The terminal $^{15}NH_3$ spectrum, however, displayed signals that followed the same trends as the ^1H ^{-15}N HSQC acquired for **1**.

Reactions of 3 (1,1/*t*,*t*-Spermidine) with GSH. (a) ^{195}Pt NMR Spectroscopy. Now that ^{195}Pt could be utilized as a fingerprint for other GSH interactions, a 1:4 **3**:GSH reaction was conducted to determine if the variation of the linker from an alkyl chain to a polyamine would affect the outcome. A control spectrum acquired for **3** (spectrum not shown) displayed a single peak at −2418 ppm. In contrast to the situation for *t*-DDP and the polynuclear drugs, the spermidine

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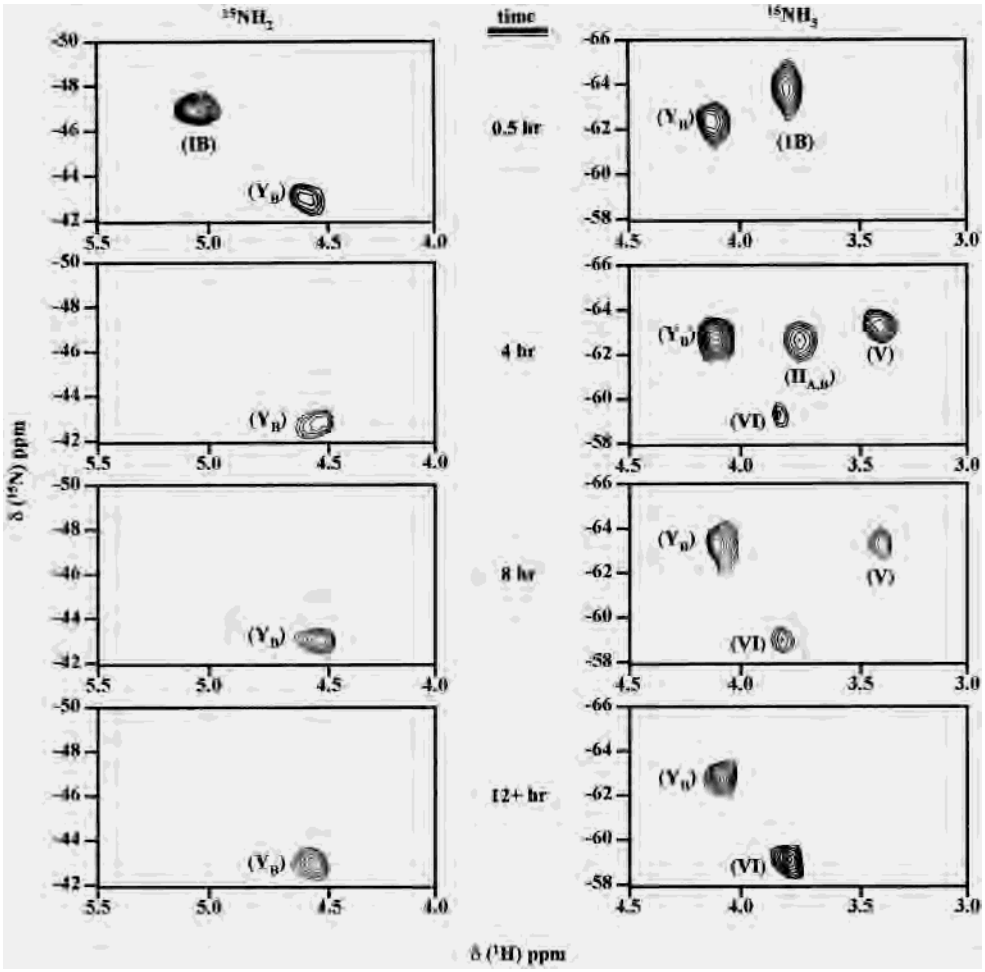

Figure 7. $^1H-^{15}N$ HSQC spectrum of the reaction of **2** with GSH (ratio 1:4) at 37 °C in DPBS (pH 7.4) from $t = 0.5$ h to $t = 12+$ h. Note that the signal for the central platinum of **2** remains constant throughout, an indication that the subunit $\mathbf{Y}_B$ is an inert species.

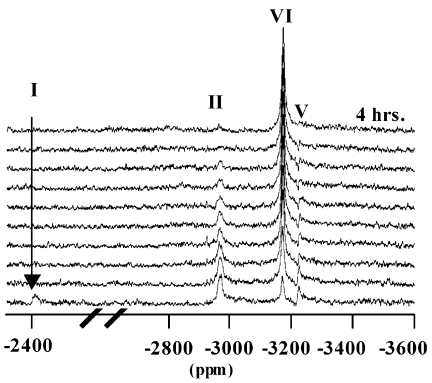

Figure 8. ^{195}Pt NMR spectra for the 1:4 reaction of **3** with GSH (120 mM DPBS, pH 7.4, 37 °C). Note that, at $t = 0.5$ h, a small fraction of unreacted drug is observed.

complex maintained a small signal in the ^{195}Pt NMR spectrum for the unreacted drug at 0.5 h. Similar to **1** and **2**, **3** exhibited three peaks (−2989, −3185, and −3226 ppm), with a constant decay of the intermediate signal at −2989 ppm over time (Figure 8). However, the formation of the final product **VI** of **3** was complete at 4 h as compared to 12+ and 7 h for *t*-DDP and the polynuclear drugs **1** and **2**, respectively.

Discussion

In the past, several investigations have been conducted on platinum drugs and the associated deactivating effects of GSH. It is known that once cellular uptake of the drug occurs, it is susceptible to interactions with the highly abundant GSH. With cellular concentrations of 5−10 mM, it is not surprising that 67% of administered cisplatin has been found to coordinate to the tripeptide.[47] In certain cell lines, resistance to cisplatin drug therapy has been directly correlated to an increase in the level of glutathione.

There was some discrepancy between the chemical shift values reported here for *trans*-$[Pt(SG)_2(NH_3)_2]$ and the bridged $[\{trans\text{-}Pt(SG)(NH_3)_2\}_2\text{-}\mu\text{-}SG]$ species and those reported previously.[21,22,36] As evidenced from the data presented here, control of pH is essential in the determination of product formation. The products identified previously by ^1H, ^{13}C, ^{195}Pt, and $^1H-^{15}N$ DEPT NMR spectroscopy of a 1:3 *t*-DDP:GSH reaction[21,22] were the same three products described in this study (**II**, **V**, and **VI**) (see Chart 3); however, buffer solutions varied from 50 mM PBS (^1H NMR spectroscopy) to 500 mM PBS (multinuclear experiments).

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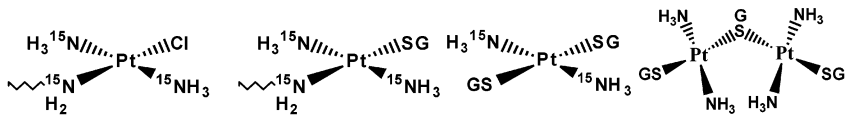
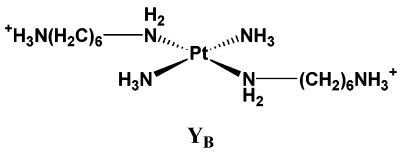
Table 1. ^{1}H and $^{15}N-^{1}H$ HSQC NMR Chemical Shifts of Platinum/Glutathione Complexes in 150 mM DPBS[a]



Drug:	NMR Nucleus[b]	Control (I)	Intermediate (II)	Trans-bis-S (V)	Bridged (VI)
t-DDP:	^{195}Pt	N/A[c]	N/A[c]	-3226	-3186
	(^{1}H / $^{15}NH_3$)	N/A[c]	N/A[c]	(3.52 / -62.7)	(3.83 / -59.1)
	^{195}Pt	-2397	-2987	-3229	-3186
1:	(^{1}H / $^{15}NH_2$)	(5.04 / -46.0)	(N/A)[e]	(N/A)[e]	(N/A)[e]
	(^{1}H / $^{15}NH_3$)	(3.87 / -63.4)	(3.77 / -62.5)	(3.53 / -62.8)	(3.85 / -59.1)
	^{195}Pt	-2417	-2990	-3229	-3185
2[d]:	(^{1}H / $^{15}NH_2$)	(5.04 / -46.0)	(N/A)[e]	(N/A)[e]	(N/A)[e]
	(^{1}H / $^{15}NH_3$)	(3.87 / -63.4)	(3.76 / -62.1)	(3.52 / -62.7)	(3.83 / -59.0)
3:	^{195}Pt	-2418	-2989	-3226	-3185

[a] Charges omitted for clarity. [b] All NMR chemical shifts reported in parts per million. [c] Due to the insolubility of *t*-DDP, spectra were not acquired of the control or intermediate species. [d] Signals for central platinum and coordinated ligands were obtained at (^{195}Pt NMR) −2648 ppm and ($^{15}N/^{1}H$) ($^{15}NH_2$) −42.8/4.58 ppm and ($^{15}NH_3$) −62.1/4.08 ppm. [e] Unresolved.

Chart 3. Chemical Structure of the Central Linking Subunit of **2**



Y_B

Under these conditions the final product of the 3:1 reaction was assigned to be [{*trans*-PtCl$(NH_3)_2$}$_2$-μ-SG]. The ^{195}Pt NMR spectrum of a solution of *trans*-[$PtCl_2(NH_3)_2$] with 3 mol equiv of GSH in 500 mM PBS (pH 6.8) afforded one broad peak at −3193 ppm, similar to the value obtained in this study for the bridged [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG]. The product of the 1:4 **1**:GSH reaction in a 100 mM PBS solution was also designated as *trans*-[$Pt(SG)_2(NH_3)_2$] with a ^{195}Pt NMR chemical shift at −3215 ppm.[48] A chemical shift value of −2811 ppm for a bridged or polymeric $PtN_{3-}S-PtN_3$ as the result of a 1:1 **1**:GSH reaction was also reported.[48] This purported species was not observed under the conditions used here. In our hands, 0.1 and 0.05 M PBS is not sufficient to maintain a pH of approximately 7.4, and it appears that, at a lower phosphate concentration, the generation of protons induced by the coordination of GSH to platinum causes a significant decrease in pH. Thus, it is evident that the pH of the reaction solution dictates whether the final product is *trans*-[$Pt(SG)_2(NH_3)_2$] (acidic pH) or [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] (pH 7.3). The results are now in agreement with those concluded by Reedijk for the $[PtCl(dien)]^+$ system.[24]

In this study, the reaction of *t*-DDP with GSH served as a control for the reactions that involved di- and trinuclear compounds (see Table 1). Evidence of the coordination of GS^- to platinum was established by the use of ^{195}Pt NMR and $^{1}H-^{15}N$ HSQC followed by HPLC coupled with ESITOFMS for the exact determination of the final product [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG]. When the reaction was carried out in a 2:3 *t*-DDP:GSH ratio, both *trans*-[$Pt(SG)_2(NH_3)_2$] and [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] were resolved via Pt NMR with an approximate 1:10 ratio, respectively. The addition of excess GSH (2:4 *t*-DDP:GSH induced a signal loss for *trans*-[$Pt(SG^-)_2(NH_3)_2$] with an associated increase in the [{*trans*-Pt(SG)$(NH_3)_2$}$_2$-μ-SG] signal. A possible mechanism of product formation is depicted in Scheme 1. The initial interaction of **I** with 2 equiv of GSH was determined to take place rapidly, in less than 30 min. A period of 7 h was determined as the time during which the *trans* influence of the coordinated sulfur liberates the *trans*-ammine of **II**, resulting in the mononuclear species **IV** and free **Y** (**III**). A number of possible routes to formation of **V** and **VI** from **II** may be envisaged. Formally, **IV** (*trans*-[$Pt(SG)(NH_3)_2X$]) is produced upon cleavage of the $Pt-NH_2$ bond; however, no evidence for this species was observed. Where X = Cl or H_2O, the species would be highly reactive in the presence of excess GSH.[49,50]

Further analysis of the HPLC data obtained from the reaction of *t*-DDP with GSH allowed the calculation of the percent area of the two peaks associated with the bis and bridged species (see Figure 1). It was proposed that the rate of formation from the bissulfur complex **V** to the bridged **VI** followed a first-order rate law with a *k* value of 0.403 s^{-1}. Although it is not possible to determine if GSH binds and causes the release of the diamine linker from **1** or **2** in a concerted or stepwise fashion, it is clear that both compounds degrade into smaller mononuclear bridged (**V**)

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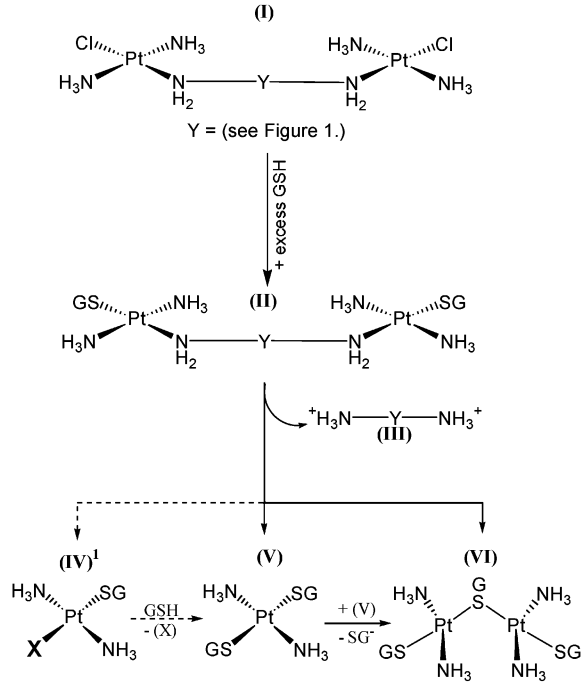
Scheme 1. Proposed Reaction Pathway in the Reaction of *t*-DDP and the Polynuclear Platinum Drugs **1**−**4** with Glutathione in Excess[a]



[a] For **IV**, X = Cl, H_2O, or $H_2N(CH_2)_6NH_3^+$. See the text for discussion.

and bissulfur (**VI**) subunits (see Table 1 for NMR peak assignments.

It has been reported that an excess of GSH is present in cancer cells;[51] thus, given the normal in vivo dose of 0.3 mg/kg for BBR3464, the ratio of **2** to GSH will far exceed 1:4. Under physiological conditions described earlier, it may be concluded that **1**, **2**, and other *trans* derivatives such as the dinuclear polyamine-bridged compounds will undergo chemical degradation upon binding of GSH. This situation is an endemic problem for all Pt−Cl-containing species, offset in the present case perhaps by enhanced DNA affinity. The results presented here show that metabolism of BBR3464 and BBR3005 may be expected to follow, in many ways, that of cisplatin and its mononuclear analogues; therapeutic efficiency will be reflected as a balance between these metabolic deactivating effects and the biological consequences of the toxic DNA adduct formation already described.[52−55]

Acknowledgment. This work was supported by operating grants from the National Institutes of Health (R01CA78754). We thank the Virginia Commonwealth University Mass Spectrometry Facility and Dr. Jason Flora for the mass spectrometry. We also thank Dr. Walter Barklage for the preparation of [^{15}N]**1**, Dr. Holger Rauter for the preparation of [^{15}N]**2**, and Dr. Alex Hegmans for the synthesis of **3**.

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