Degradation of Bidentate-Coordinated Platinum(II)-Based DNA Intercalators by Reduced L-Glutathione

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We have examined the interaction of [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**1**, 56MESS), [(5-methyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**2**, 5MESS), [(5,6-dimethyl-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)platinum(II)]$^{2+}$ (**3**, 56MERR), and [(5,6-dimethyl-1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (**4**, 56MEEN) with reduced L-glutathione and L-methionine. Both thiols degrade all four complexes, mainly by displacing the ancillary ligand and forming a doubly bridged dinuclear complex. The degradation half-life of all the complexes with methionine is >7 days, indicating that these reactions are not biologically relevant. The rate of degradation by glutathione appears to be particularly important and shows an inverse correlation to cytotoxicity. The least active complex, **4** ($t_{1/2}$ glutathione: 20 h), degrades fastest, followed by **3** (31 h), **2** (40 h), and **1** (68 h). The major degradation product, [bis-μ-{reduced L-glutathione}bis{5,6-dimethyl-1,10-phenanthroline}bis{platinum(II)}]$^{2+}$ (**5**, 56MEGL), displays no cytotoxicity and is excluded as the source of the anticancer activity. Once bound by glutathione, these metal complexes do not then form coordinate bonds with guanosine. Partial encapsulation of the complexes within cucurbit[*n*]urils is able to stop the degradation process.

Introduction

Over the last 10 years we have developed a family of over 60 platinum(II)- and 1,10-phenanthroline-based DNA intercalators, some of which demonstrate enormous potential in the treatment of human cancers that show resistance to current chemotherapeutic agents.[1–6] With these DNA intercalators we have shown that ancillary ligand chirality and phenanthroline functional group substitution play an important role in determining their cytotoxicity; methylation in the 5, or 5 and 6, positions on the phenanthroline ligand and the ancillary ligand in an *S*,*S*-conformation, particularly with the 1,2-diaminocyclohexane ligand, confers the highest cytotoxicity.[1–6] Although it is believed that DNA is the ultimate target for these metal complexes, DNA-binding experiments have shown no significant differences in the DNA adducts formed between many of the DNA intercalators.[4] This suggests that all DNA adducts formed by this family of metal complexes are similarly cytotoxic and/or that DNA binding may not be the sole mechanism that determines their cytotoxicity.[4] We have hypothesized that differing cellular uptake levels or differing intracellular drug transport may determine how much of each DNA intercalator reaches its target intact, thereby affecting cytotoxicity.[4]

The tripeptide reduced L-glutathione (γ-glutamylcysteinylglycine, GSH, Figure 1) is abundant in cells at concentrations between 0.5 and 10 mM[7] and is involved in many cellular processes, including metabolism, protection of cells from oxidative stress, and detoxification and/or bioactivation of drugs.[8–10] L-Methionine is an essential amino acid that contains a thioether group capable of binding to platinum atoms (Figure 1). The anticancer drugs cisplatin and [(*trans*-PtCl(NH_3)$_2$)$_2$(μ-*trans*-Pt(NH_3)$_2$(NH_2(CH_2)$_6$$NH_2$)$_2$)]($NO_3$)$_4$) (**6**, BBR3464), the latter an agent capable of overcoming cisplatin resistance *in vitro*/*in vivo*,[10,11] are readily degraded into nonactive complexes by both glutathione and methionine.[10,11] This degradation occurs through the ability of the sulfur atoms to displace both the chloride and monodentate am(m)ine ligands of cisplatin and **6**. This degradation leads to decreased levels of active drug reaching its cellular target, DNA, and modulation of the drug's inter- and intracellular transport.

Importantly, glutathione and methionine are unable to displace bi- or tridentate amine ligands (like ethylenediamine and diethylenetriamine), which is why these ligands are routinely used in thiol protein degradation experiments.[12,13] Their inertness limits the number of degradation products formed and thus simplifies spectral analysis.[14–16] To date there have been no papers describing the removal of bi- or tridentate amine ligands from platinum complexes by thiols or thioethers.

In this study we report the unexpected degradation by reduced L-glutathione, and to a lesser extent the degradation by L-methionine, of four platinum(II)-based DNA intercalators, [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)-platinum(II)]$^{2+}$ (**1**, 56MESS), [(5-methyl-1,10-phenanthroline)-(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**2**, 5MESS), [(5,6-dimethyl-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)platinum(II)]$^{2+}$ (**3**, 56MERR), and [(5,6-dimethyl-1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (**4**, 56MEEN) (Figure 1). The degradation process and products were examined using ^{1}H and ^{195}Pt NMR[a], electrospray ionization mass spectrometry, and *in vitro* L1210 murine leukemia growth inhibition assays.

Results

The platinum(II)-based DNA intercalator complexes **1**, **2**, **3**, and **4** (1 mM) were reacted with 4 mM reduced L-glutathione

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[a] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; ESI, electrospray ionization mass spectrometry; **1**, [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$; **2**, [(5-methyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$; **3**, [(5,6-dimethyl-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)platinum(II)]$^{2+}$; **4**, [(5,6-dimethyl-1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$; **5** [bis-μ-{reduced L-glutathione}bis{5,6-dimethyl-1,10-phenanthroline}bis{platinum(II)}]$^{2+}$; NOESY, nuclear Overhauser effect spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

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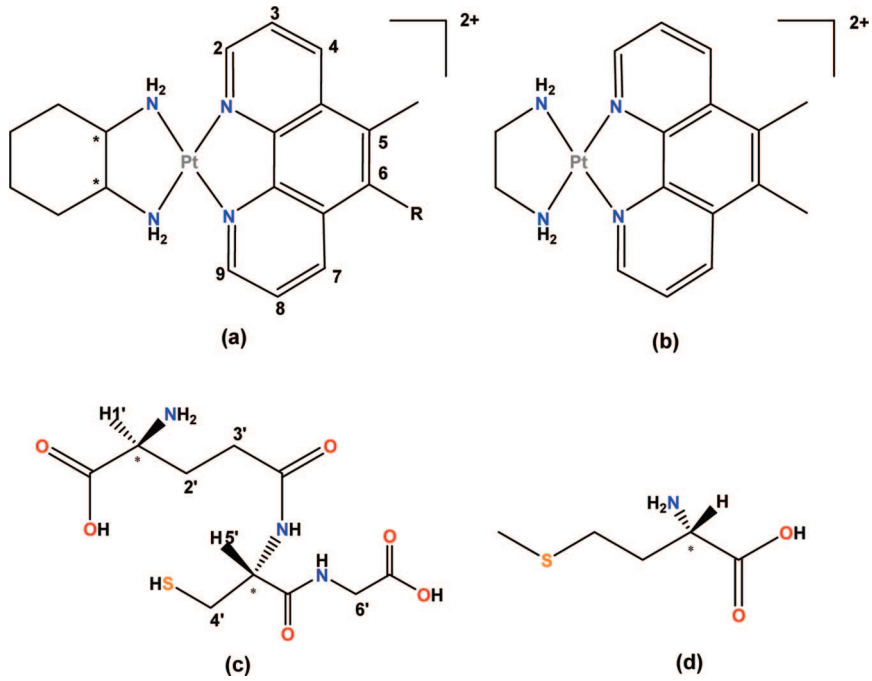

Figure 1. Chemical structures of (a) this family of platinum(II)-based DNA intercalators: R = CH_3 (**1**, **3**), R = H (**2**); (b) **4**; (c) reduced L-glutathione; and (d) L-methionine, showing the proton-numbering scheme used. An asterisk (*) indicates a chiral center, either *R* or *S*. Anions have been omitted for clarity but are generally chlorides.

(glutathione) or L-methionine (methionine) at 37 °C in either unbuffered D_2O or phosphate-buffered saline (pH 7) in D_2O for periods of up to 7 days. All four DNA intercalators are reactive toward glutathione and methionine, as indicated by a color change from either a clear pink (**2**), cream (**3**, **4**), or pale yellow (**1**) solution to a dark brown solution, with the formation after approximately 3 days of an orange precipitate, which increases in amount as the reaction proceeds. The precipitate is not soluble in aqueous solvents and shows only limited solubility in hot dimethylsulfoxide, which excluded its analysis by standard chemical instrumentation.

The reaction of all four metal complexes with methionine is very slow ($t_{1/2}$ > 7 days), indicating that the metal complexes' reactions with this amino acid *in vitro*/*in vivo* are probably not relevant to the structure–activity relationship of this family of DNA intercalators. As such, there was no further examination of their reaction kinetics or products. Reactions with glutathione, however, appear to be particularly important. All four metal complexes react with glutathione at differing rates and with what appears to be an inverse correlation with their *in vitro* cytotoxicity in the L1210 murine leukemia cell line (paired students *t* test, $P < 0.05$). Complex **4**, the least active anticancer DNA intercalator, reacts the fastest ($t_{1/2}$, 20 h), followed by **3** ($t_{1/2}$, 31 h), **2** ($t_{1/2}$, 40 h), then **1** ($t_{1/2}$, 68 h), in the order corresponding to their increasing cytotoxicity (Table 1). The reaction half-lifes of the metal complexes were not signficantly different whether straight D_2O or phosphate-buffered saline (pH 7) D_2O was used, indicating that drug degradation is not due to a decrease in the pH of the solution as protons are lost from the thiol.

^{1}H NMR Analysis of the Reduced L-Glutathione−Metal Complex Reaction Solutions. The reaction of all four metal complexes with glutathione and methionine is also observed by the appearance, in ^{1}H NMR spectra, of new metal complex resonances in the aromatic region and new glutathione resonances in the aliphatic region. As an indicative example, the ^{1}H NMR spectra of complex **4** shows just two doublet resonances and one doublet of doublets resonance at the beginning of the reaction (Figure 2). After 24 h, another, nonsymmetrical set of phenanthroline resonances (four doublet and two doublet of doublets resonances) is observed and can be assigned as the major degradation product. After 48 h a third set of nonsymmetrical phenanthroline resonances is clearly resolvable, which are assigned as a minor degradation product.

Table 1. Cytotoxicity (L1210 murine leukemia cancer cell line) and Reduced L-Glutathione Degradation Half-Lives of Four Platinum(II)-Based DNA Intercalators[a]

metal complex	IC_{50} (μM)	$t_{1/2}$ (h)
cisplatin	1.00	3.3[b]
1	0.0092[c]	68
2	0.033[d]	40
3	0.46	31
4	1.50	20

[a] Cytotoxicity values represent IC_{50} in μM, which is defined as the required concentration of DNA intercalator to induce 50% inhibition of cell growth, and were determined from at least three experiments. Half-lives ($t_{1/2}$, h) represent the time taken for 50% of the DNA intercalators (1 mM) to react with reduced L-glutathione (4 mM) at 37 °C and were determined from at least three experiments. [b] Data taken from ref 20. [c] Data taken from ref 5. [d] Data taken from ref 4.

For all four metal complexes the aromatic resonances corresponding to free metal complex also move downfield as the reaction progresses. This result is consistent with a decrease in free metal complex concentration, as the chemical shift of intercalator resonances in aqueous solvents has been shown previously to change with varying intercalator concentration.[17] The chemical shift varies due to $\pi-\pi$ interactions between stacked intercalator molecules; as the concentration varies, the number of $\pi-\pi$ interactions changes, with greater molecular stacking inducing ^{1}H downfield resonance shifts and fewer $\pi-\pi$ interactions inducing upfield shifts.

^{195}Pt NMR Analysis of the Reduced L-Glutathione−Metal Complex Reaction Solutions. The ^{195}Pt NMR of the reaction mixture of complexes **1**/**3** and glutathione reveals two resonances: one large peak at −2828 ppm, consistent with the

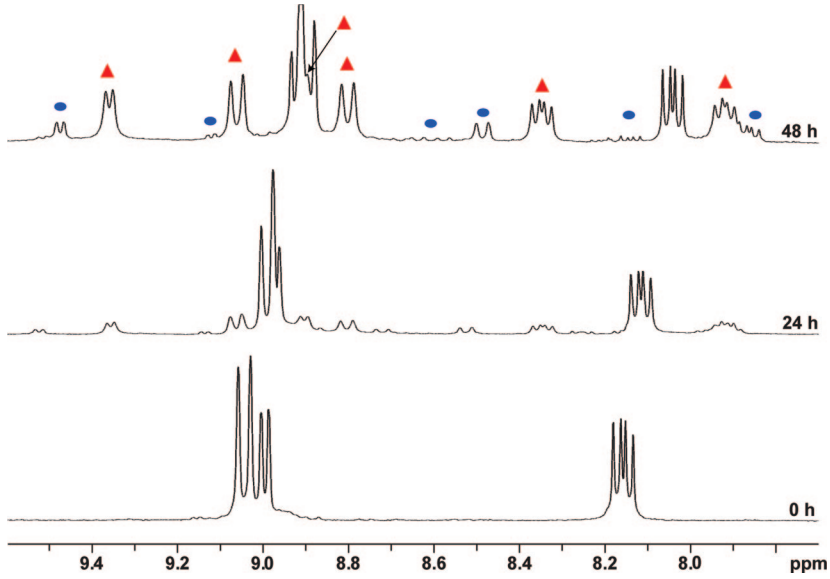

Figure 2. ^{1}H NMR spectra of the reaction of **4** (1 mM) with reduced L-glutathione (4 mM) at 37 °C showing the production of the major degradation product (▲) **5** and other minor product(s) (●). These spectra are indicative of those obtained for **1** and **3**. The spectra of **2** are considerably more complicated due to the lack of phenanthroline symmetry because of the presence of only a single methyl group.

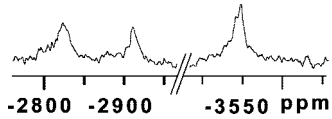

Figure 3. ^{195}Pt NMR spectrum of the reaction mixture of complex **2** 72 h after reaction with 4 molar equiv of reduced L-glutathione, showing unreacted **2** (−2828 ppm), its doubly bridged dinuclear platinum(II) degradation product, **5** (−2916 ppm), and a proposed mononuclear degradation product at −3551 ppm.

unreacted N_4 coordination sphere of complexes **1/3**, and another resonance slightly upfield at −2890 ppm, consistent with a dinuclear platinum degradation product bridged by two individual glutathione molecules.[13,14] In some spectra, a very small resonance is also observed at −3509 ppm (>1%). Similar to **1**, complex **4** also gives two resonances at −2828 and −2916 ppm, which are the free metal complex and a doubly bridged dinuclear complex, respectively. The ^{195}Pt NMR spectrum of complex **2** however gives three distinct resonances (Figure 3). The first at −2828 ppm is consistent with unreacted **2**, and the second resonance, at −2916 ppm, is again consistent with a doubly bridged dinuclear platinum degradation product. A third resonance appears ∼600 ppm upfield from free **2** at −3551 ppm and is consistent with a N_3S coordination sphere.[18,19] The degradation resonances of all four metal complexes increase in intensity as the reactions proceed and can account for between 20% and 60% of the total platinum in the solutions, but are always comparable in relative size to the degradation resonances observed in the equivalent ^{1}H NMR spectra.

Electrospray Ionization Mass Spectrometry Analysis of the Reduced L-Glutathione−Metal Complex Reaction Solutions. The ESI+ mass spectra of all four metal complexes after reaction with glutathione gave ion peaks between 200 and 1400 *m*/*z*, consistent with a number of platinum degradation products. As an indicative example, the ESI+ mass spectrum of **1** $[M - H^+]^+$ showed the free drug as an ion peak at 516.4 *m*/*z*. A major degradation product peak occurred at 709.5 *m*/*z*, consistent with a 2+ bisplatinum complex containing two phenanthroline ligands and two individual bridging glutathione ligands (degradation product complex **5**, Figure 4). This peak could also mask the peak of a mononuclear platinum degradation product where one glutathione molecule displaces a diaminocyclohexane (dach) ligand and binds the platinum atom through two coordination sites. Another major ion isotope peak occurs at 823.6 *m*/*z*, which indicates the presence of a 1+ complex containing platinum, phenanthroline, diaminocyclohexane, and glutathione, indicating that the dach is still attached, possibly via one amine group and the glutathione is bound through a single site, possibly through the sulfur group (degradation product complex **7**). A further minor ion peak at 615.5 *m*/*z* can be assigned to a degradation product where the phenathroline ligand has been removed and replaced by a single glutathione molecule (degradation product complex **8**).

Synthesis of [Bis-*μ*-{reduced L-glutathione}bis{5,6-dimethyl-1,10-phenanthroline}bis{platinum(II)}]$^{2+}$(5). The metal complex was synthesized by first reacting an equimolar concentration of K_2PtCl_4 and 5,6-dimethyl-1,10-phenanthroline in dimethylsulfoxide. The chloride ligands were removed from the resultant crystals by the addition of $AgNO_3$ in dimethylformamide, before an equimolar amount of reduced L-glutathione was added. The desired product was purified by precipitation with diethyl ether then eluted on a 2 g C18 reverse phase column with a 50:50 mixture of acetonitrile and water.

Complex **5** was characterized using ^{1}H (one-dimensional Watergate and two-dimensional nuclear Overhauser effect spectroscopy and double quantum correlated spectroscopy) and ^{195}Pt NMR, ESI+ mass spectrometry, and elemental analysis. In the ^{1}H NMR spectrum, there are two doublet resonances at 9.31 and 8.75 ppm assigned as the phenanthroline H2/H9 protons, the resonances at 8.96 and 8.66 ppm are assigned as the H4/H7 protons, and two doublet of doublets resonances at 8.25 and 7.75 ppm are assigned as the H3/H8 protons (Figure 5). Two phenanthroline methyl singlet resonances appear in the aliphatic region at 2.72 and 2.64 ppm. There appears to be two sets of glutathione resonances that overlap, giving the appearance of mutliplets. Finally, the glycine and cysteine amine resonances have shifted significantly in **5** compared to free reduced L-glutathione; in the Watergate ^{1}H NMR spectrum of complex **5** the glycine −NH resonance has shifted downfield from 8.42 to 8.62 ppm and the cysteine −NH resonance has shifted upfield from 8.37 to 8.18 ppm.

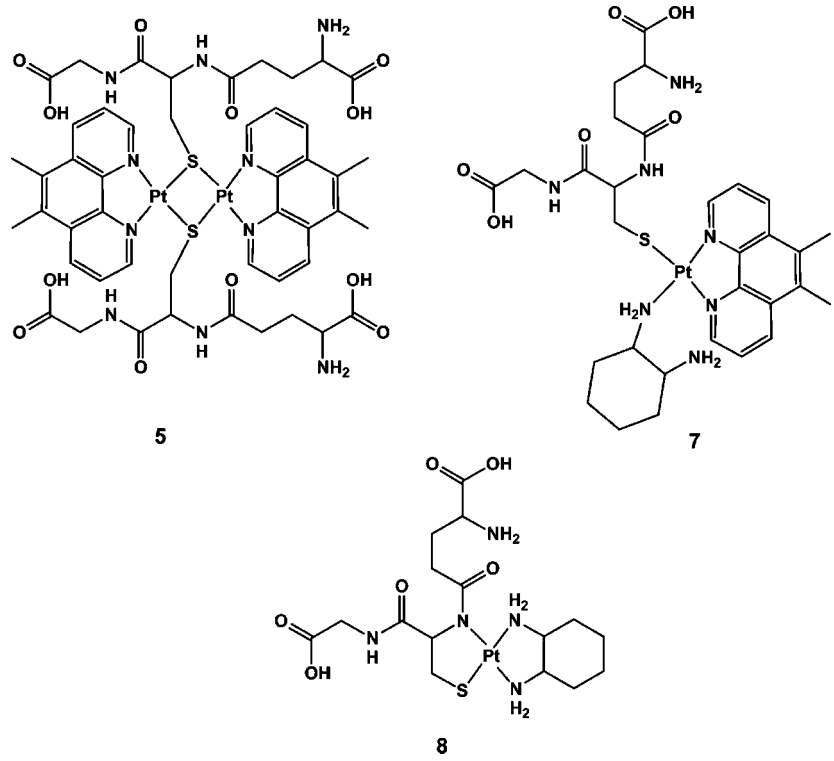

Figure 4. Proposed chemical structures of the three degradation products observed from the reaction of **1** with reduced L-glutathione from the analysis of the electrospray ionization mass spectrum. Charges, counterions, and the chirality of bonds have been omitted for clarity.

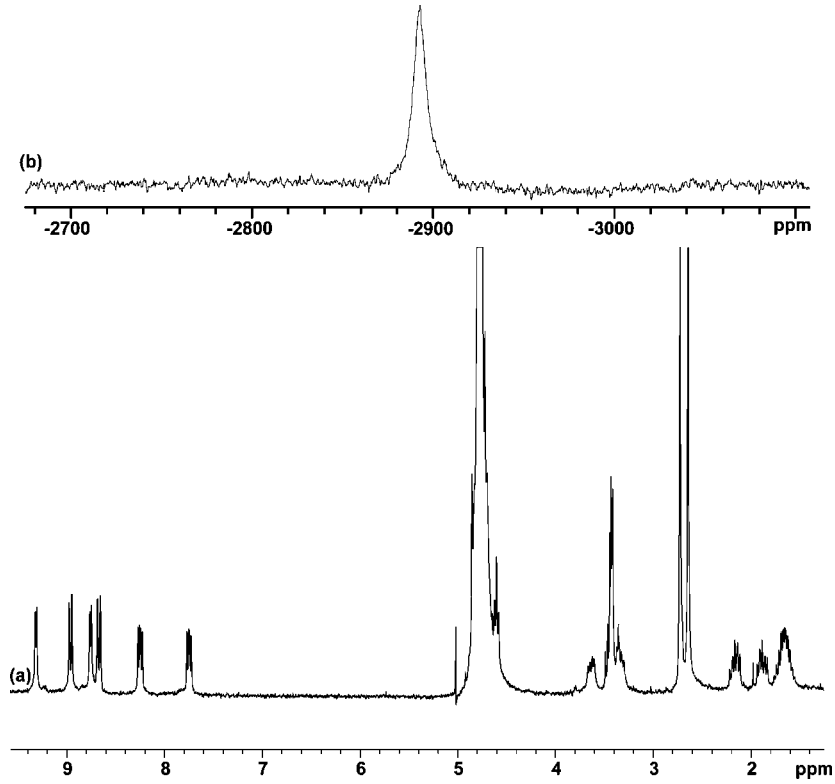

Figure 5. (a) ^{1}H and (b) ^{195}Pt NMR spectra of the major reduced L-glutathione degradation product of this family of platinum(II)-based DNA intercalators, complex **5**. The ^{1}H NMR spectrum of **5** is identical to the major degradation product indicated in Figure 2.

Normally, the phenanthroline ligand has only two doublet resonances and one doublet of doublets resonance in the aromatic region and only one methyl resonance in the aliphatic region. The ^{1}H NMR spectrum of complex **5** therefore indicates that the metal complex is not symmetrical. Previously, Papadia et al. have described a dinuclear platinum(II) complex containing two phenanthroline ligands, which is bridged by two individual glutathione molecules binding through the sulfur groups (i.e., **5** but without the phenanthroline methyl groups).[14] This metal complex can exist as two different isomers with the glycine residues either in a *syn* or *anti* position to each other (Figure 6).[14] The ^{1}H

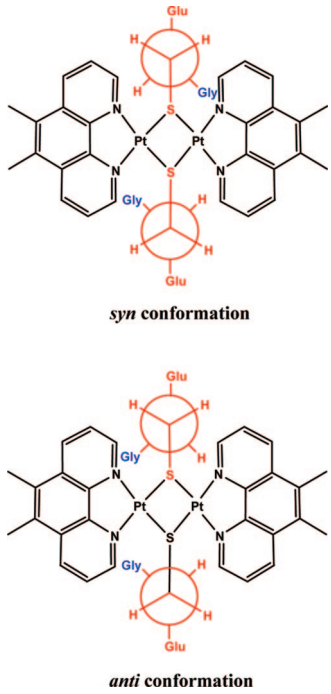

Figure 6. Proposed chemical structure of **5** showing the two possible isomers (*syn* and *anti*), with respect to the glycine residue. These isomers disrupt the symmetry of the dinuclear complex, thereby giving two sets of phenanthroline and glutathione resonances in the ^{1}H NMR spectrum. This figure is a modified version of the one given in ref 14.

NMR spectrum of **5** is therefore consistent with a mixture of isomers of a doubly bridged dinuclear platinum(II) complex.

The ^{195}Pt NMR and ESI+ mass spectra are also consistent with the proposed chemical structure. A single ^{195}Pt NMR resonance is observed at −2893 ppm, similar to that reported by Papadia et al. at −2871 ppm (see Figure 5).[14] In the ESI+ mass spectrum, complex **5** shows a single major ion peak (100%) at 709.1 *m*/*z*. The isotopic pattern of the ion peak is consistent with the theoretical isotopic distribution for a doubly bridged dinuclear platinum complex ($C_{48}H_{55}N_{10}O_{12}Pt_2S_2^{+}$, data not shown), which further confirms the proposed chemical structure of **5**.

Reaction of 5 with 5′-Guanosine Monophosphate. 5′-Guanosine monophosphate (2 mM) and **5** (1 mM) were incubated at 37 °C in unbuffered D_2O (600 μL), and the ^{1}H NMR spectrum was recorded at intervals for up to 7 days. There was no change in either the line width or chemical shift of the guanosine H8 or H1′ resonances nor the proton resonances of complex **5**, indicating that the reduced L-gluthathione ligands are not displaced by guanosine.

Reaction of the Metal Complexes with Reduced L-Glutathione in the Presence of Cucurbit[*n*]uril. All four metal complexes (1 mM) were incubated with cucurbit[*n*]uril ($n = 6$, 7, or 8) (1 mM) and reduced L-glutathione (4 mM) in unbuffered D_2O (600 μL) at 37 °C, and ^{1}H NMR spectra were recorded. No metal complex degradation products were observed in any spectra at intervals of up to 7 days.

Discussion

In this study we have examined the reaction of four metal complexes from a family of platinum(II)-based DNA intercalator complexes, **1**, **2**, **3**, and **4**, with reduced L-glutathione (glutathione) and L-methionine (methionine). These four metal complexes were chosen because they display cytotoxicities ranging from **1**, which is up to 100-fold more active than cisplatin, to **4**, which is less cytotoxic than cisplatin (see Table 1). Complex **1** is also considerably more active than cisplatin in several human cancer cell lines. As such, these four metal complexes allowed us to examine the structure–activity relationship of thiol degradation in this family of metal complexes. These metal complexes differ mostly in the structure and chirality of their ancillary ligands. **1** and **2** both contain 1*S*,2*S*-diaminocyclohexane as the ancillary ligand, **3** contains the same ligand but with different chirality (i.e., *R*,*R*), and **4** contains the achiral ligand ethylenediamine.

Unexpectedly, all four metal complexes react with glutathione and to a lesser extent with L-methionine. This result is unexpected because bi- and tridentate amine ligands have previously been shown to be inert to removal by thiols and thiolates, regardless of the other coordinating ligands.[12] This is in contrast to cisplatin and **6**, which contain only monodentate chloride and am(m)ine ligands and readily undergo subsitution reactions with both types of thiols.

The reaction of the metal complexes with methionine does not appear to be biologically relevant, as the half-lifes are greater than 7 days. Their reaction with glutathione, however, appears to be particularly relevant and shows an inverse correlation to their cytotoxicity in the L1210 murine leukemia cell line (see Table 1). The time difference in reaction half-lifes is also significant; complex **1** is more than 2-fold slower to react with glutathione than **4**. The reaction of all four metal complexes with glutathione, however, is considerably slower than cisplatin or **6** (6- to 20-fold compared to cisplatin).[20] Despite the relatively long degradation half-lifes, we conclude that the reaction of these DNA intercalators with glutathione is biologically relevant, as platinum drugs have been shown to have long circulating lifetimes in the human body. In humans only half of the drug dose is excreted within 9 days for oxaliplatin,[21] 5.8 days for carboplatin,[22] and 5.4 days for cisplatin.[22] A reliable human *in vivo* half-life of **6** is yet to be reported, but a recent phase I clinical trial found that **6** was still being excreted 15 days after treatment.[23]

It is unclear why these DNA intercalators degrade at differing rates. It is possible that the chirality of **3**, or lack of chirality in the case of **4**, allows a chiral-specific interaction with one or more amino acids of the glutathione, whereas for complexes **1** and **2** the interaction may be less favorable. Such an interaction may stabilize a **3** or **4** complex that is longer-lived than the **1**− or **2**−glutathione interactions, or the chirality of **1/2** may provide steric bulk that prevents glutathione from more closely approaching the platinum atom. Certainly steric factors do play a role in sulfur attack on platinum centers, and one drug currently undergoing clinical trials, *cis*-[$PtCl_2$(2-methylpyridine)NH_3] (picoplatin), was designed specifically so that the methyl group sits directly over the platinum atom, providing steric hindrance to sulfur attack.[24] Unfortunately no chiral-specific interactions between the free ligands *S*,*S*- or *R*,*R*-dach and glutathione are observed. At concentrations of 4 mM there are no significant changes in the ^{1}H NMR proton resonances of either the diaminocyclohexane compounds or glutathione when combined. There are no intermolecular cross-peaks in two-dimensional rotating frame Overhauser effect NMR spectra.

Examination of the reaction products of the metal complexes with glutathione using ^{1}H and ^{195}Pt NMR and mass spectrometry reveals the formation of a number of degradation products. For all four metal complexes the major degradation product appears to be a dinuclear platinum complex with two phenanthroline

ligands and two individual glutathione molecules coordinated to the platinum atoms through their sulfur groups, forming a doubly bridged complex, **5** (see Figure 4). Formation of a doubly bridged dinuclear platinum complex is consistent with the product observed by del Socorro Murdoch et al. from the reaction of [Pt(en)Cl_2] with reduced glutathione.[13] Mass spectrometry also revealed the formation of other minor degradation species. From both the ^{1}H and ^{195}Pt NMR and ESI+ mass spectra we tentatively assigned the most relevant minor product as a mononuclear species either where the ancillary ligand has been fully displaced by a single glutathione molecule that is bound through the cysteine sulfur and nitrogen atoms or where the ancillary ligand has been partially displaced by a glutathione molecule that is bound solely through its sulfur group (i.e., degradation product complex **7** in Figure 4). Both minor degradation products would be expected to have nonsymmetrical phenanthroline resonances and an N_3S coordination sphere yielding a ^{195}Pt NMR resonance of around 3500 ppm, consistent with the resonance observed at −3550 ppm in Figure 3. While both of these observed metal complexes may be minor degradation products in their own right, both may also simply be intermediates in the formation of **5**.

To further examine the major degradation product, **5** was synthesized and purified separately. Complex **5** has ^{1}H and ^{195}Pt NMR spectra identical to the major degradation product of **1** and an ESI+ mass spectrum showing a single peak with isotope patterning consistent with a $C_{48}H_{55}N_{10}O_{12}Pt_2S_2^+$ (i.e., [**5** − H^+]$^+$) ion.

It is possible that much of the cytotoxicity of this family of DNA intercalators is derived not from the metal complexes themselves, but from their degradation products. To test this hypothesis, **5** was examined for cytotoxicity in the L1210 murine leukemia cell line. Complex **5** displays no growth inhibition at concentrations up to 50 μM. Also of significant interest is the ability of thiols to act as reservoirs for platinum(II)-based drugs inside the cell. Cisplatin is easily degraded by both glutathione and methionine, but when degraded by glutathione can still go on to form coordinate bonds at the N7 position of guanosine.[12] It was therefore of interest to determine whether the anticancer activity of this family of metal complexes arises not from DNA intercalation but from a cisplatin-like coordinate adduct with DNA formed from their glutathione degradation products. To test this hypothesis, **5** was incubated with 5′-guanosine monophosphate for time periods of up to 7 days and the ^{1}H NMR spectrum of the solution recorded at intervals. At no stage was there evidence of coordinate adduct formation to guanosine (as would have been indicated by downfield shifts of the guanosine H8 and H1′ proton resonances), suggesting that these metal complexes do not act by forming coordinate covalent adducts with DNA through their degradation products.

The glutathione reaction half-lifes of the four metal complexes, taken together with the cytotoxicity and guanosine binding results of **5**, suggest two things: (a) the major degradation product of this family of DNA intercalators is not the cause of their cytotoxicity, and (b) if degradation by glutathione can be slowed, or even prevented, then the metal complexes' cytotoxicity and efficacy can be further increased.

Previously, we have examined the use of cucurbit[*n*]urils (where $n = 5$, 6, 7, 8, or 10) as drug delivery vehicles (Figure 7).[5,17,25,26] Cucurbit[*n*]urils (where $n = 6$, 7, or 8) were generally found to bind over the metal complexes' ancillary ligands (diaminocyclohexane or ethylenediamine) with a variety of platinum(II)-based DNA intercalators.[5,17] This encapsulation was found to modulate the cytotoxicity of the metal complexes, with

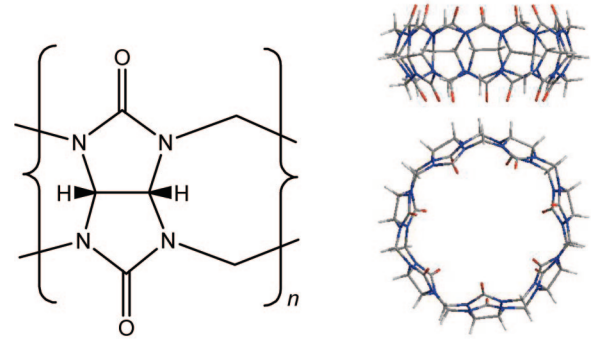

Figure 7. Chemical structure of cucurbit[*n*]uril (where $n = 5$, 6, 7, 8, or 10) and a model of cucurbit[7]uril demonstrating the barrel shape and internal cavity of cucurbit[*n*]urils within which platinum(II)-based drugs can be partially encapsulated and slowly released, preventing their degradation by thiol amino acids, peptides, and proteins.

cucurbit[6]uril increasing the cytotoxicity of some of the DNA intercalators. Given these results, we were interested to see if cucurbit[*n*]uril could prevent metal complex degradation by glutathione. When all four metal complexes were incubated with 1 molar equiv of cucurbit[*n*]urils (where $n = 6$, 7, or 8) and 4 molar equiv of glutathione, no degradation was observed in the ^{1}H NMR spectra for time periods up to 7 days. This result is consistent with similar experiments where cucurbit[*n*]uril was able to reduce the rate of reaction of mono- and multinuclear platinum(II)-based anticancer complexes with biological nucleophiles (e.g., L-cysteine and guanosine).[25,27,28] We are now examining cucurbit[*n*]uril derivatives, different macrocycles, and other molecular structures as drug-delivery vehicles for not just this family of platinum(II)-based DNA intercalators but also for other platinum(II)-based anticancer complexes that are of interest.[5,29,30]

Conclusions

This family of platinum(II)-based DNA intercalator complexes is unexpectedly degraded by reduced L-glutathione, and to a lesser extent by L-methionine, where the loss of the ancillary (either ethylenediamine or 1,2-diaminocyclohexane) or intercalating ligand (5-methyl-1,10-phenanthroline or 5,6-dimethyl-1,10-phenanthroline) forms a mixture of stereoisomer mono- and dinuclear reaction products. The major degradation product is a dinuclear platinum complex with two phenanthroline ligands linked through the sulfur atoms of two individual reduced L-glutathione molecules. While the degradation of the DNA intercalators by methionine does not appear to be biologically relevant (half-lives >7 days), degradation by glutathione occurs in a metal complex specific manner. This degradation appears to be inversely correlated with the cytotoxicity of the platinum complexes, where the most active complexes degrade at the slowest rate and the less active complexes degrade at the fastest rate. The proposed major degradation product, **5**, demonstrated no cytotoxicity up to 50 μM and thus is not the cause of the cytotoxicity of this family of platinum(II)-based DNA intercalators. The results therefore indicate that if degradation of these platinum(II)-based DNA intercalators can be further slowed or even stopped, then the cytotoxicity and efficacy of the already highly active anticancer DNA intercalator complexes, such as **1** and **2**, could be further increased.

Experimental Section

Materials. The platinum(II) complexes **1**, **2**, **3**, and **4** were synthesized as previously described.[5] Cucurbit[6, 7, and 8]urils were

provided by Dr. Anthony Day, UNSW@ADFA, and made as previously described.[31] L-Methionine was purchased from Sigma. Reduced L-glutathione, 5,6-dimethyl-1,10-phenanthroline, 5-methyl-1,10-phenanthroline, ethylenediamine, 1*R*,2*R*-diaminocyclohexane, and 5′-guanosine monophosphate were purchased from Sigma-Aldrich. 1*S*,2*S*-Diaminocyclohexane was purchased from Fluka. $AgNO_3$ was purchased from BDH Chemicals. Deuterated solvents D_2O (99.9%) and d_7-DMF (99.9%) were purchased from Cambridge Isotope Laboratories. All general solvents were used as provided and were of analytical grade or better.

Nuclear Magnetic Resonance. One- and two-dimensional NMR spectra were obtained on either a 300 MHz Varian Mercury spectrometer or a 400 MHz Bruker Advance 400 spectrometer, in D_2O, referenced internally to the solvent. ^{195}Pt NMR were externally referenced to K_2PtCl_4 (−1631 ppm; D_2O, 25 °C, Ξ = 21.4 MHz) at an operating frequency of 85.88 MHz.[32,33] ^{1}H NMR were run at 37 °C unless otherwise specified. For one-dimensional spectra, a spectral width of 7000 Hz was used with 50 000 data points and a relaxation delay of 1 s. Two-dimensional nuclear Overhauser effect spectra (NOESY) were obtained using a 5000 Hz spectral width with 256 increments in the t1 dimension, 2048 points in the t2 dimension, and a mixing time of 0.35 s. Rotating frame Overhauser effect spectra were recorded with the same parameters as the NOESY, but with a mixing time of 0.5 s. Two-dimensional double quantum correlated spectroscopy experiments were recorded over a spectral width of 5000 Hz using 256 increments in the t1 dimension, 2048 points in the t2 dimension, and a pulse repetition delay of 3.0 s. All chemical shifts are reported in parts per million (ppm).

Electrospray Ionization Mass Spectrometry. Positive ion ESI mass spectra were acquired using either a Micromass Quattro Micro spectrometer or a Micromass Qtof2 spectrometer. Both spectrometers were equipped with Z-spray probes and were used to obtain spectra of solutions containing samples with concentrations ranging between 10 and 50 μM, which were injected into the instruments at a flow rate of 10 μL min^{-1}. For the Quattro Micro spectrometer the source and desolvation temperatures were 150 and 120 °C, respectively, while for the Qtof2 spectrometer these values were 60 and 150 °C, respectively. The capillary tip potential and cone voltage were 2500 and 50 V, respectively, for the Quattro Micro spectrometer, and for the Qtof2 spectrometer these values were 2500 and 100 V. For both instruments between 10 and 50 acquisitions were summed to obtain spectra, which were calibrated against a standard CsI solution (750 mM) over the same *m*/*z* range. Theoretical isotopic distributions were determined using Mass Spec Calculator Professional v4.09.

Growth Inhibition Assays. The murine leukemia cancer cell line L1210 was grown in RPMI-1640 medium supplemented with 5% fetal bovine serum. Standard conditions for cell maintenance were used: 37 °C humidified incubator with 5% CO_2. Cells were plated in 96-well plates (100 μL per well) at a concentration of 4 × 10^4 cells mL^{-1} in complete medium and then treated with a range of concentrations of drug in 100 μL of medium (0.00013 to 50 μM). The cells were incubated for 48 h under standard conditions before drug cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-based growth inhibition assay. The assay was carried out by adding 50 μL of MTT solution (5 mg mL^{-1}) to the cell/drug mixtures and incubating for 2 h in standard conditions. The cells were harvested and mixed with 200 μL of DMSO before the optical density (OD_{590}) was read on a plate reader (MultisKan EX, LabSystems, Finland). Absorbance values at each drug concentration were plotted (*y*-axis) against the corresponding concentration of drug (*x*-axis), and the IC_{50} values were calculated from the resulting dose−response curve. Data given are averages derived from between two and six independent experiments tested in duplicate or triplicate.

Time Course Reactions. The metal complexes (1 mM) were reacted with either reduced L-glutathione or L-methionine (4 mM) in unbuffered D_2O or phosphate-buffered saline (NaCl 140 mM, Na_2HPO_4 8 mM, pH 7) in D_2O at 37 °C, and the ^{1}H NMR spectra were obtained over a period of 7 days. All reactions were performed in triplicate. The time course experiments were also conducted under the conditions stated above but with the addition of cucurbit[*n*]uril (where *n* = 6, 7, or 8) (1 mM). Half-lives ($t_{1/2}$, h), defined as the time taken for 50% of the DNA intercalator to react with glutathione/methionine, were determined graphically by plotting elapsed time (h) versus percentage of unreacted species, measured from the change in relative integration of the nondegraded metal complex aromatic peaks.

Synthesis of [Bis-μ-{reduced L-glutathione}bis{5,6-dimethyl-1,10-phenanthroline}bis{platinum(II)}]$^{2+}$ Chloride Hydrate (5). [$PtCl_2$(5,6-dimethyl-1,10-phenanthroline] (0.24 g, 0.5 mmol)[6] in dimethylformamide (DMF, 25 mL) was added to a solution of $AgNO_3$ (0.17 g, 1.0 mmol) in DMF (25 mL) and the resulting solution stirred overnight in the dark. The resultant precipitated AgCl was removed using a 0.45 μm filter to yield a clear yellow filtrate. A solution of reduced L-glutathione (0.15 g, 0.5 mmol) in DMF (5 mL) was added, and the resulting solution stirred overnight in the dark. Diethyl ether (300 mL) was added to precipitate a pale yellow product, which was collected by vacuum filtration and washed with diethyl ether. The yellow solid was stirred in water for 1 h. The insoluble impurities were removed by gravity filtration, and the filtrate was loaded onto a Waters 2 g C18 reverse phase Sep-Pak, removing unreacted glutathione with water. The product was then eluted using acetonitrile−water (50:50) as a yellow solution and lyophilized. Anal. Calcd for $C_{48}H_{56}Cl_2N_{10}O_{12}Pt_2S_2 \cdot 7H_2O$: C, 35.67; H, 4.37; N, 8.67. Found: C, 35.26; H, 3.98; N, 8.28. ^{1}H NMR (300 MHz, D_2O): δ 9.31 (d, J = 5.1 Hz, 2H, H2/H9), 8.96 (d, J = 8.5 Hz, 2H, H4/H7), 8.75 (d, J = 5.2 Hz, 2H, H2/H9), 8.66 (d, J = 8.5 Hz, 2H, H4/H7), 8.25 (dd, J = 5.2, 3.4 Hz, 2H, H3/H8), 7.75 (dd, J = 5.2, 3.4 Hz, 2H, H3/H8), 4.60 (t, J = 6.4 Hz, 2H, H5′), 3.63 (m, 2H, H6′), 3.44 (m, 2H, H6′), 3.32 (m, 2H, H1′), 2.72 (s, 6H, CH_3), 2.64 (s, 6H, CH_3), 2.16 (m, 4H, H4′), 1.90 (m, 4H, H3′), 1.66 (m, 4H, H2′). ^{195}Pt NMR (85 MHz, D_2O): δ −2893 ppm (bs). ESI-MS: *m*/*z* calcd for $C_{48}H_{56}N_{10}O_{12}Pt_2S_2^+$, 1418.28; found, 1418.0 $[M - 2H]^{2+}$, 709.1 $[M - H]^+$.

Reaction of 5 with 5′-Guanosine Monophosphate. 5′-Guanosine monophosphate (2 mM) and **5** (1 mM) were incubated at 37 °C in 600 μL of unbuffered D_2O, and ^{1}H NMR spectra were obtained at time periods of up to 7 days.

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