Towards a Better Understanding of the Cisplatin Mode of Action

Vicente Marchán,[a] Virtudes Moreno,[b] Enrique Pedroso,[a] and Anna Grandas*[a]

Abstract: We have studied how platinum(II) complexes [Pt(dien)Cl]Cl, [Pt(en)Cl_2] and cisplatin react with hybrid molecules that contain sulfur and nitrogen ligands, in particular Phac-Met-linker-p5′dG (Phac = phenylacetyl), Phac-His-linker-p5′dG, Phac-His-Met-linker-p5′dG and Phac-His-Gly-Met-linker-p5′dCATGGCT. The progress of the reactions was monitored by HPLC, and by [^{1}H,^{15}N]-HSQC NMR when ^{15}N-cisplatin was used. The products were isolated and characterised by using enzymatic and chemical reactions and spectroscopic techniques (UV and/or NMR spectroscopy, electrospray or MALDI-TOF mass spectrometry). The combined use of digestion with proteases and reaction with hydrogen peroxide followed by mass spectrometric analysis indicated the platinum coordination positions on the peptide moiety of the largest hybrid. Monofunctional Pt–S adducts were transformed into Pt–N complexes in which Pt–N7 bonds were formed preferentially. Most of the chelates isolated had Pt–S bonds, and, in the case of cisplatin complexes, loss of the ammine *trans* to sulfur gave rise to the formation of tricoordinate species with platinum-mediated peptide–nucleotide cross-links. 1,2-Intrachain platinum GpG adducts were only obtained in very small amounts (1–4%).

Keywords: cisplatin · nucleic acids · peptide–oligonucleotide hybrids · peptides · platinum

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)], by far one of the most studied metal complexes, is a highly effective anticancer drug presently in clinical use, although it is not without side effects. Its therapeutic effect is believed to result from the formation of a macrochelate with two adjacent guanines in a DNA chain.[1]

On the basis of competition studies with monofunctional platinum complexes ([Pt(dien)]$^{2+}$; dien = 2-aminoethyl-1,2-ethanediamine) which have shown that platinum may migrate from the sulfur atom of methionine to the N7 of guanine, it has been suggested that proteins might act as a reservoir of cisplatin and thus mediate its transfer to DNA.[2] Studies with peptides that contain histidine and methionine have shown that [Pt(dien)]$^{2+}$ can migrate from sulfur to imidazole,[3] and that the bifunctional [Pt(en)]$^{2+}$ (en = 1,2-ethanediamine) complex can form macrochelates in which the metal is coordinated to sulfur and one of the imidazole nitrogen atoms.[4] Finally, our own studies on the reaction of cisplatin with a histidine–deoxyguanosine nucleopeptide have shown that platinum can also simultaneously coordinate the N7 of guanine and either the Nπ or Nτ imidazole ring atoms.[5, 6]

To get a deeper insight into the actual behaviour of cisplatin we were interested in assembling the different pieces of the puzzle together in one molecule. To this end we synthesised,[7] besides the simplest hybrid models Phac-Met-linker-p5′dG (**1**) (Phac = phenylacetyl) and Phac-His-linker-p5′dG (**2**), two peptide–(oligo)nucleotide hybrids that contain histidine, methionine and deoxyguanosine: Phac-His-Met-linker-p5′dG (**3**), and Phac-His-Gly-Met-linker-p5′dCATGGCT (**4**). We report here on the study of the reaction of these hybrids with [Pt(dien)]$^{2+}$ and [Pt(en)]$^{2+}$, as well as with the actual anticancer drug, cisplatin.

Results

Aqueous solutions of the hybrids were mixed with aqueous solutions of the platinum(II) complexes, and, unless otherwise indicated, the reactions were carried out without any further pH adjustment. [Pt(en)Cl_2] and cisplatin were solubilised by heating the aqueous suspension at 90 °C for 10–15 min, which is known to cause partial hydrolysis of cisplatin.[8]

Complexation reactions of hybrid 1: The reactions of the Pt^{II} complexes with hybrid **1** are summarised in Scheme 1. The reaction of **1** with [Pt(dien)]$^{2+}$ and [Pt(en)]$^{2+}$ gave the expected results:[2, 9] platinum migrated from sulfur (**1a**) to the guanine N7 (**1b**) in the first case, and the [Pt(en){(**1**)-S,N7}]$^+$ chelate (**1c**) was formed in the second case.

[a] Prof. A. Grandas, V. Marchán, Prof. E. Pedroso
Departament de Química Orgànica
Facultat de Química, Universitat de Barcelona
Martí i Franquès 1–11, 08028 Barcelona (Spain)
Fax: (+34) 93-33-97-878
E-mail: grandas@qo.ub.es

[b] Prof. V. Moreno
Departament de Química Inorgànica
Facultat de Química, Universitat de Barcelona (Spain)

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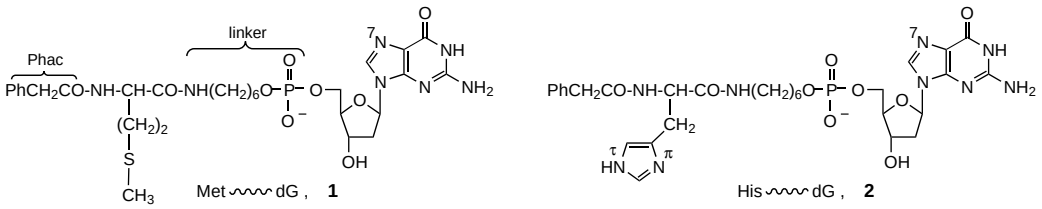
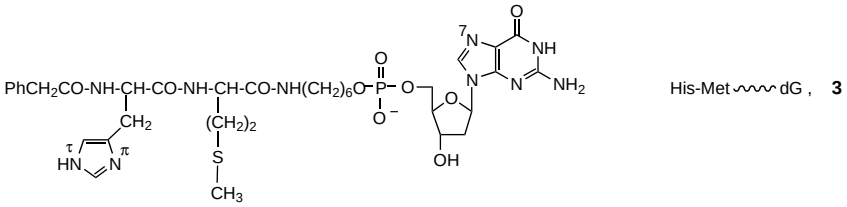
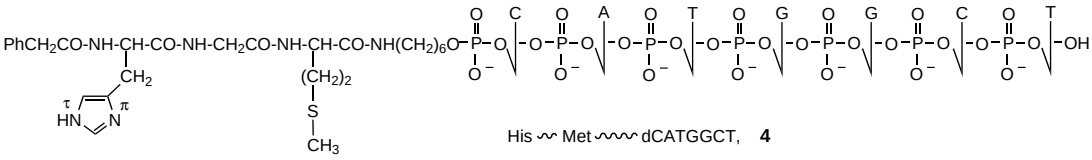
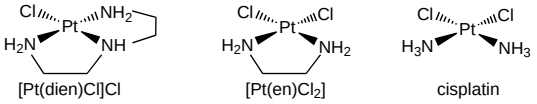






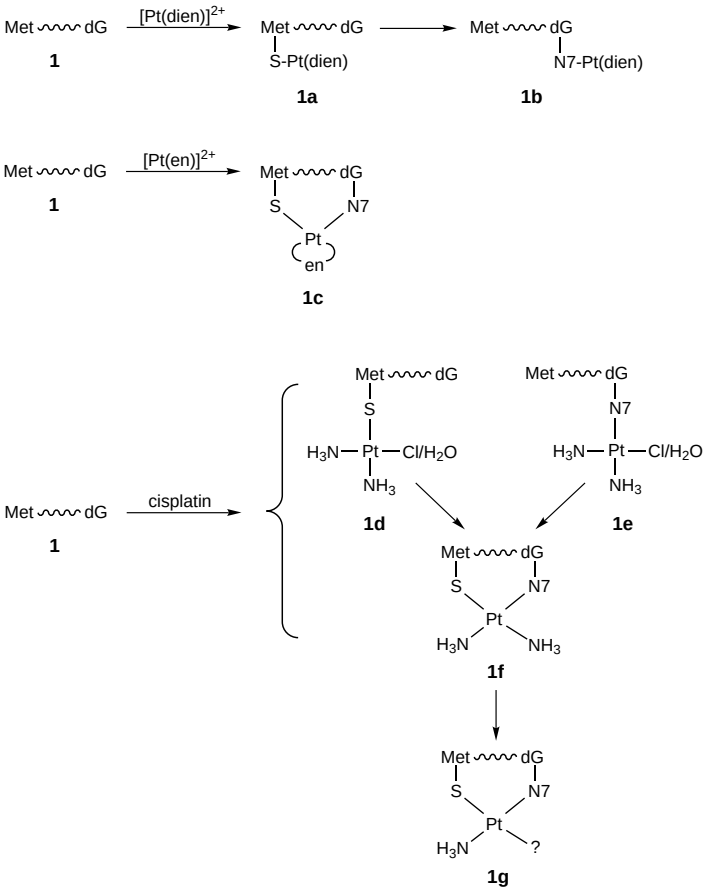


Three products were detected at the beginning of the reaction of **1** with cisplatin: the monofunctional Pt–S adduct (**1d**), the monofunctional Pt–N7 adduct (**1e**), and the Pt–S,N7 chelate (**1f**). As the reaction proceeded, **1d** and **1e** disappeared and a new product (**1g**) was formed. Adduct **1g** was identified, by mass spectrometry and ^{1}H and [^{1}H,^{15}N]-HSQC NMR experiments, as a Pt–S,N7 chelate in which the ammine *trans* to the sulfur atom had been lost. We could not identify which ligand replaced the ammine group, but this position might be occupied by the O6 of guanine, or by a water molecule, which, in turn, might establish a hydrogen bond with the O6 atom of the guanine base.[10] Adduct **1g** did not react with 5′dGMP (5′-monophosphate of 2′-deoxyguanosine).

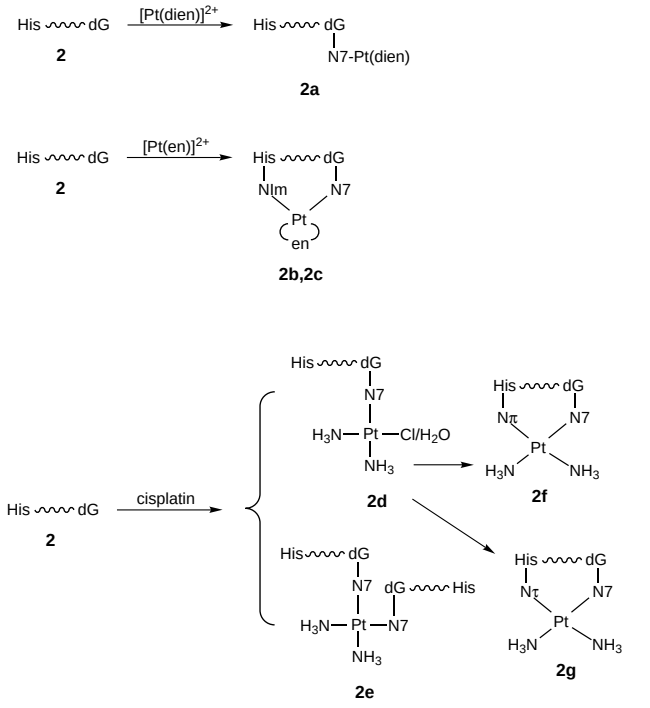
Complexation reactions of hybrid 2: The reactions of the Pt^{II} complexes with hybrid **2** are summarised in Scheme 2. $[Pt(dien)]^{2+}$ reacted only with the N7 atom of **2** to give adduct **2a**. Two chelates were obtained when **2** reacted with $[Pt(en)]^{2+}$, in which platinum was coordinated to the N7 of guanine and one of the imidazole nitrogen atoms (**2b**,**2c**).

The reaction of **2** with cisplatin initially yielded four products, the monofunctional Pt–N7 complex **2d**, a bifunctional complex in which the metal was linked to the N7 atoms of two hybrids **2e**, and the two chelates **2f** (Pt–N7,Nπ) and **2g** (Pt–N7,Nτ). The relative proportions changed as the reaction progressed, until the complete disappearance of **2d**. Neither the monofunctional nor bifunctional Pt–N7 complex was obtained in an analogous previous experiment,[6] but that reaction had been carried out at slightly basic pH. The reaction of **2** with cisplatin at pH 8 gave rise to the formation of only **2f** and **2g**.

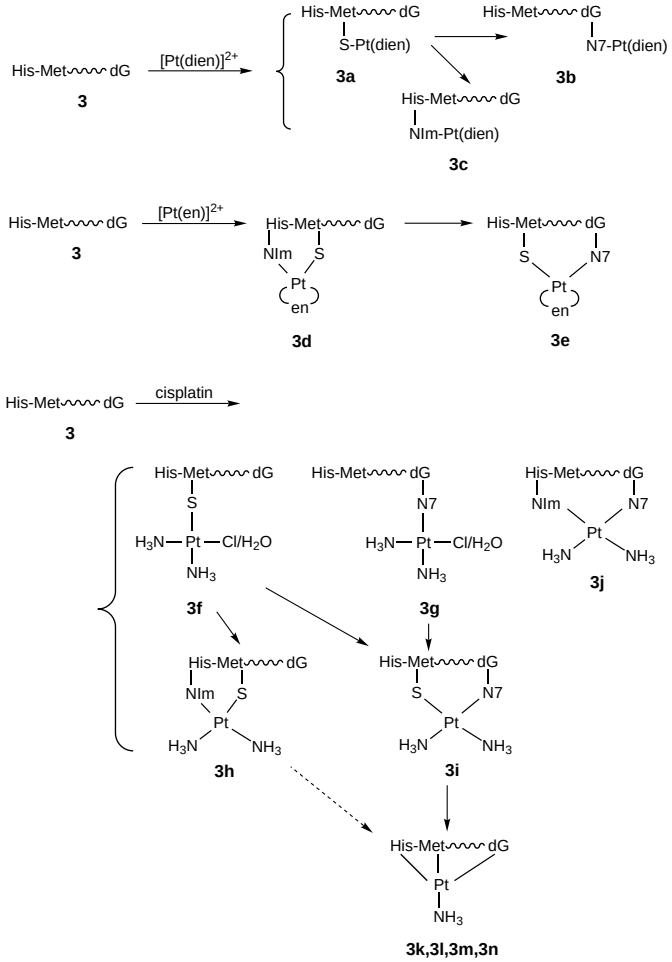
Complexation reactions of hybrid 3: The reactions of the Pt^{II} complexes with hybrid **3** are summarised in Scheme 3. Hybrid **3** was almost immediately and quantitatively transformed into



Scheme 1. Reactions of the Pt^{II} complexes with hybrid **1** (Phac-L-Met-linker-5′dG).



Scheme 2. Products formed in the reaction of hybrid **2** (Phac-L-His-linker-p5′dG) with the Pt^{II} complexes.



Scheme 3. Reactions between hybrid **3** (Phac-L-Met-L-His-linker-p5′dG) and the three Pt^{II} complexes.

the Pt–S complex **3a** upon reaction with $[Pt(dien)]^{2+}$. From **3a**, the Pt–N7 (**3b**) and Pt–NIm (**3c**; Im = imidazole, NIm = any of the imidazole nitrogen atoms) complexes were formed, complex **3b** in larger amounts. The reaction between **3** and $[Pt(en)]^{2+}$ (Figure 1 and Figure 2) afforded an intermediate (**3d**) that evolved into the Pt–S,N7 chelate (**3e**). Complex **3d** had the mass of a chelate ($[Pt(en)(\mathbf{3})]^{+}$), its UV spectrum (λ_{max}, shape) showed that platinum was not linked to the guanine N7 and it did not react with H_2O_2, an indication of Pt–S coordination. These data suggested that **3d** could be either the Pt–S,N^{α}(Met) or the Pt–S,NIm complex. It has been reported[4] that the reaction of histidine- and methionine-containing peptides with $[Pt(en)]^{2+}$ yields both types of complex and that, depending on the reaction conditions, either both are formed simultaneously or the Pt–S,N^{α}(Met) complex is formed first. Since we did not detect Pt–S,N^{α}(Met) chelates in any other reaction, while we have evidence of the formation of the Pt–S,NIm complex (**4e**) in the case of **4** (see below), we conclude that **3d** is probably the Pt–S,NIm chelate. In any case, transformation of **3d** into **3e** must have occurred by cleavage of a Pt–N bond and formation of the Pt–N7 bond. Such surprising behaviour would mean that Pt^{II} could migrate to guanine not only from a thioether group but also from either the N^{α} of methionine or from the side chain of histidine.

Cisplatin gave rise to a much more complex mixture upon reaction with **3** (Figure 1 and Figure 2), and some of the trends previously found with **1** and **2** were reproduced. Many products were detected over the first 4 h, which, on the basis of UV spectroscopy, MS, chemical behaviour (stability throughout the reaction process or to reaction with H_2O_2) and [^{1}H,^{15}N]-HSQC NMR data were inferred to be the Pt–S and Pt–N7 monofunctional complexes **3f** and **3g**, respectively, and the Pt–S,NIm (**3h**), Pt–S,N7 (**3i**) and Pt–NIm,N7 (**3j**) chelates. Chelate **3i** was the main product at a reaction time of 4 h (more than 50%), and its disappearance was accompanied by the formation of the main final products. After 16 h, signals corresponding to the ammine *trans* to the sulfur atom were no longer detected. When a stationary situation was reached (after 3 days), the main products present in the reaction mixture (**3k**–**3n**) were isolated and characterised. All products have the same molecular formula, which corresponds to $[Pt(NH_3)(\mathbf{3})]^{+}$, and have a UV bathochromic shift. These data, together with the information from the CH_3, H8, H2 and H5 ^{1}H chemical shifts, suggest that in **3k**–**3n** platinum is coordinated to one ammine, and to methionine, histidine and guanosine. Since the metal can be linked to either the Nπ or the Nτ imidazole nitrogens, and two diastereomers may originate from coordination to sulfur, four isomers can be formed.

Complexation reactions of hybrid 4: The reactions of the Pt^{II} complexes with hybrid **4** are summarised in Scheme 4. Before we began this study, we examined the reaction between the oligonucleotide moiety of hybrid **4**, 5′dCATGGCT, and the three platinum(II) complexes. The reaction with $[Pt(dien)]^{2+}$ afforded a product which was platinated at the 5′G nucleobase, and the reactions with $[Pt(en)]^{2+}$ and cisplatin gave, as expected, the intrachain Pt–N7/5′G,N7/3′G chelates.

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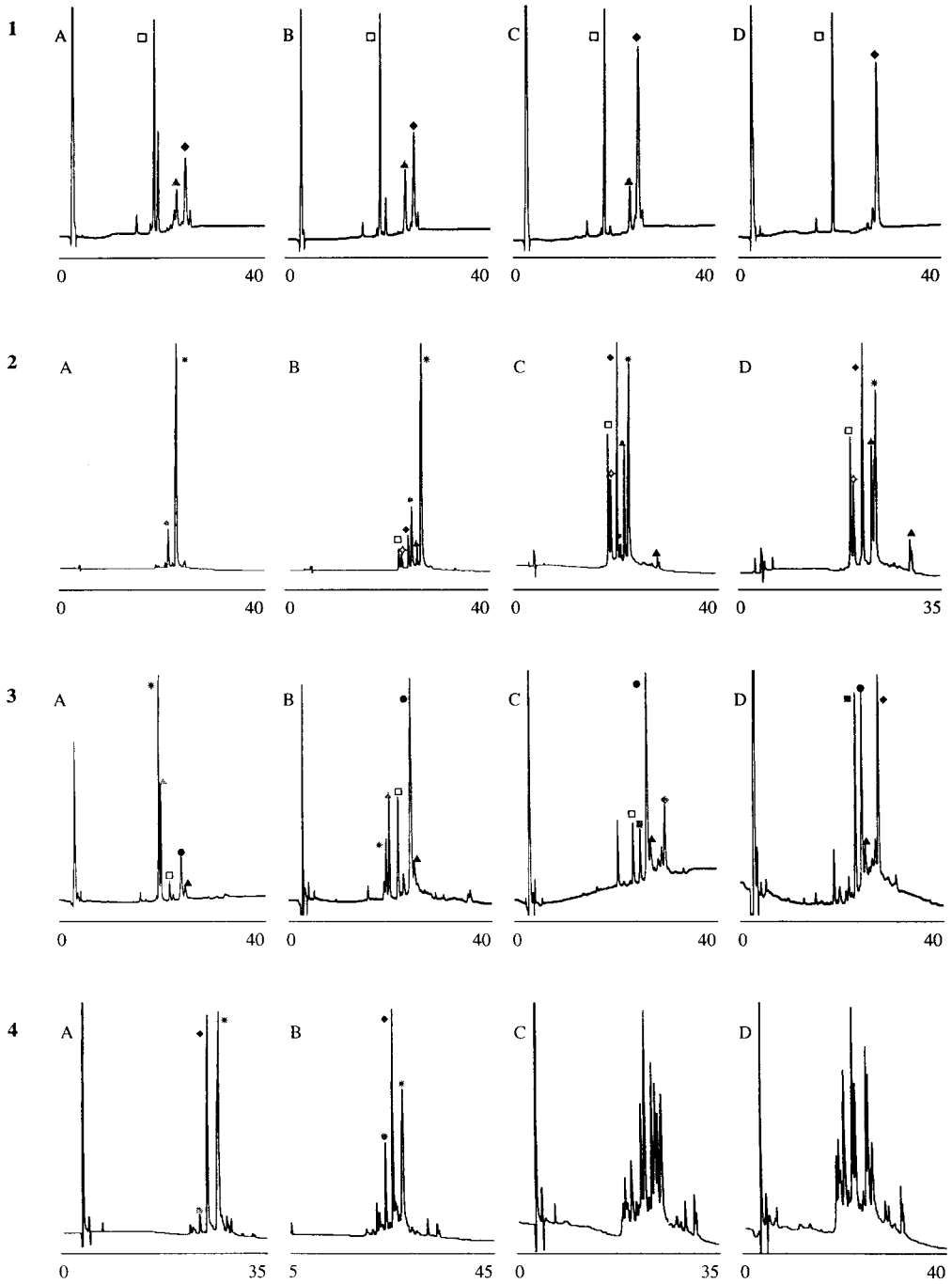

Figure 1. HPLC profiles of the reaction mixtures at different reaction times. 1) **3** and $[Pt(en)]^{2+}$, A: 1 h, B: 1 h 45 min, C: 6 h, D: 5 days; 2) **4** and $[Pt(en)]^{2+}$, A: 7 min, B: 45 min, C: 5 h 30 min, D: 3 days; 3) **3** and cisplatin: A: 1 h, B: 3 h 30 min, C: 24 h, D: 3 days; 4) **4** and cisplatin: A: 20 min, B: 1 h 45 min, C: 8 h, D: 24 h.

Two new products were quickly formed upon reaction of **4** with $[Pt(dien)]^{2+}$, the Pt–S complex (**4a**) and the Pt–N7/5′G complex (**4b**). As the reaction progressed, two doubly platinated species appeared in which two $[Pt(dien)]^{2+}$ units were linked to the 5′G nucleobase and either methionine or histidine, complexes **4c** and **4d**, respectively. At the beginning of the process (0–2 h) **4a** predominated over **4b**, whereas at 48 h the relative proportion was reversed.

Six products different from **4** were detected throughout its reaction with $[Pt(en)]^{2+}$ (Figures 1 and 2). Platinum was coordinated to the side chains of histidine and methionine (**4e**) in the first main chelate formed, while in the other chelates coordination was established with the imidazole nitrogens and either the N7 of 5′G (**4f**, **4g**) or that of 3′G (**4h**, **4i**). The product resulting from coordination to the two guanine residues (**4j**) was detected (4%) only after 48 h. Hence, **4** only partially reproduces the behaviour of the simpler model **3**. In both cases the Pt–S,NIm chelate was formed, but the evolution of the reaction with $[Pt(en)]^{2+}$ was different. Hybrid **3** gave the Pt–S,N7 chelate (**3d**), whereas the main products found upon reaction with **4** were the Pt–NIm,N7 macrochelates (**4f**–**4i**).

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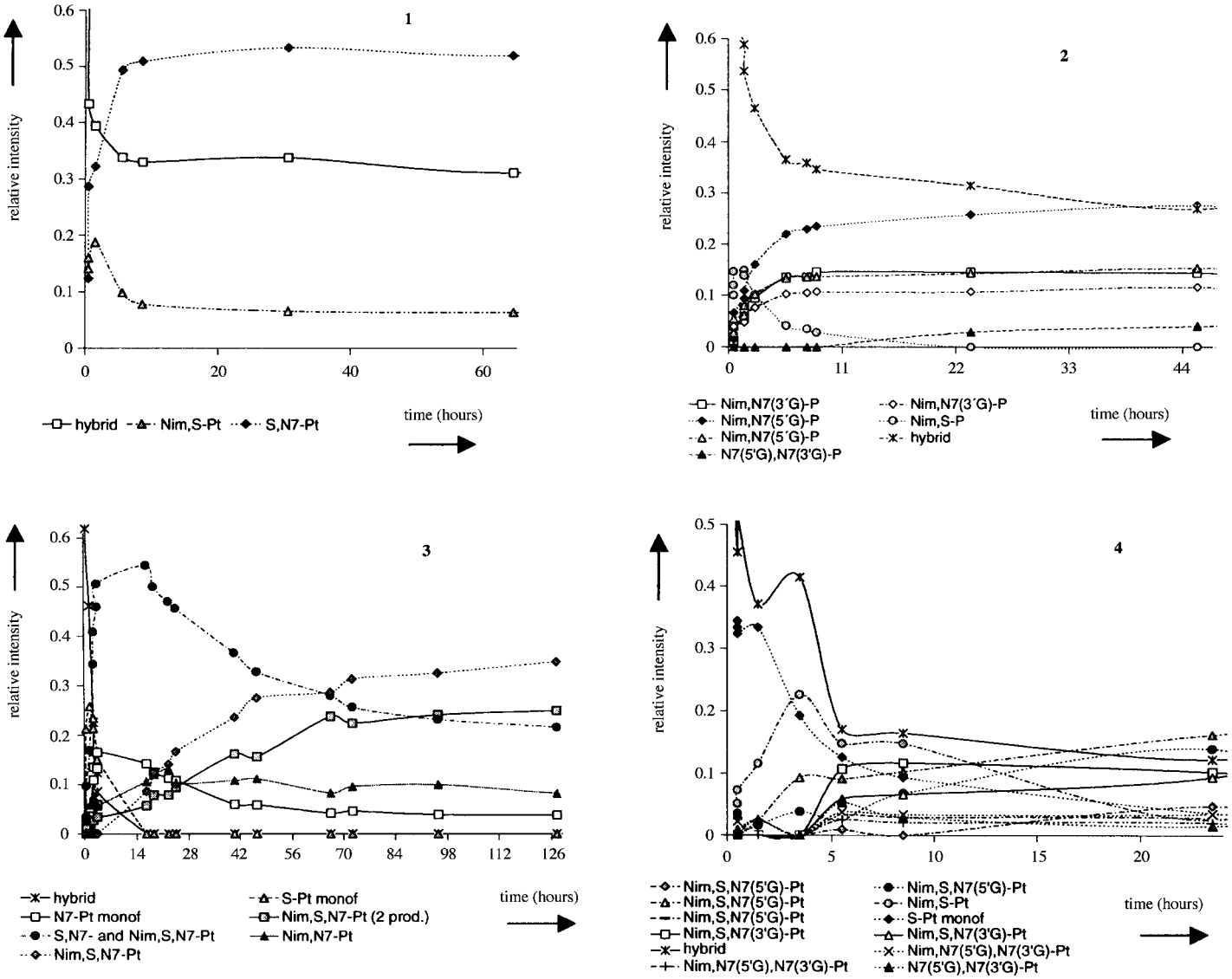

Figure 2. Time dependence of species formation for the reactions 1) **3** and $[Pt(en)]^{2+}$, 2) **4** and $[Pt(en)]^{2+}$, 3) **3** and cisplatin and 4) **4** and cisplatin.

As with **3**, HPLC monitoring of the reaction of **4** with cisplatin showed that a very complex process was taking place (Figure 1 and Figure 2). The two main products formed at the beginning of the reaction were identified as the Pt–S monfunctional adduct (**4k**) and the Pt–S,NIm chelate (**4l**). Adduct **4k** predominated at short reaction times, then decreased as the concentration of **4l** increased. Both then subsequently disappeared. After 9 h, signals corresponding to ammine *trans* to sulfur were no longer detected, and at 24 h the stationary situation was virtually reached. Isolation of the different final products was difficult. In at least four of the products obtained (**4m**–**4p**) platinum was coordinated to one ammine, sulfur, one of the imidazole nitrogen atoms and the N7 of the 5′G nucleobase. Two products were isolated (minor proportion) which only differed in that coordination was established with the 3′G nucleobase (**4q**, **4r**). In a third group of complexes (**4s**, **4t**) platinum was coordinated to one ammine, histidine and the two guanines. Finally, we isolated a product (1.4%) in which two ammine groups and the two guanines were linked to platinum (**4u**).

Discussion

The kinetic preference of platinum for sulfur ligands and the preference for the 5′G in GG sequences found in the above-described experiments agree with previously described results.[2, 11] The preference of the metal for the N7 of guanine rather than the nitrogen atoms of the imidazole ring is also clear.

The most important conclusions that can be drawn from the above-described results concern the use of $[Pt(dien)]^{2+}$ and $[Pt(en)]^{2+}$ as models of the monofunctional products formed upon reaction with cisplatin and of cisplatin, respectively.

The previously described migration of $[Pt(dien)]^{2+}$ from the sulfur of methionine to the N7 of guanine or to the imidazole nitrogens was reproduced in this study. However, when the methionine-containing hybrids reacted with cisplatin, formation of Pt–S monofunctional complexes was followed by formation of chelates, which, in turn, yielded tricoordinate products that in most cases still maintained the Pt–S bond

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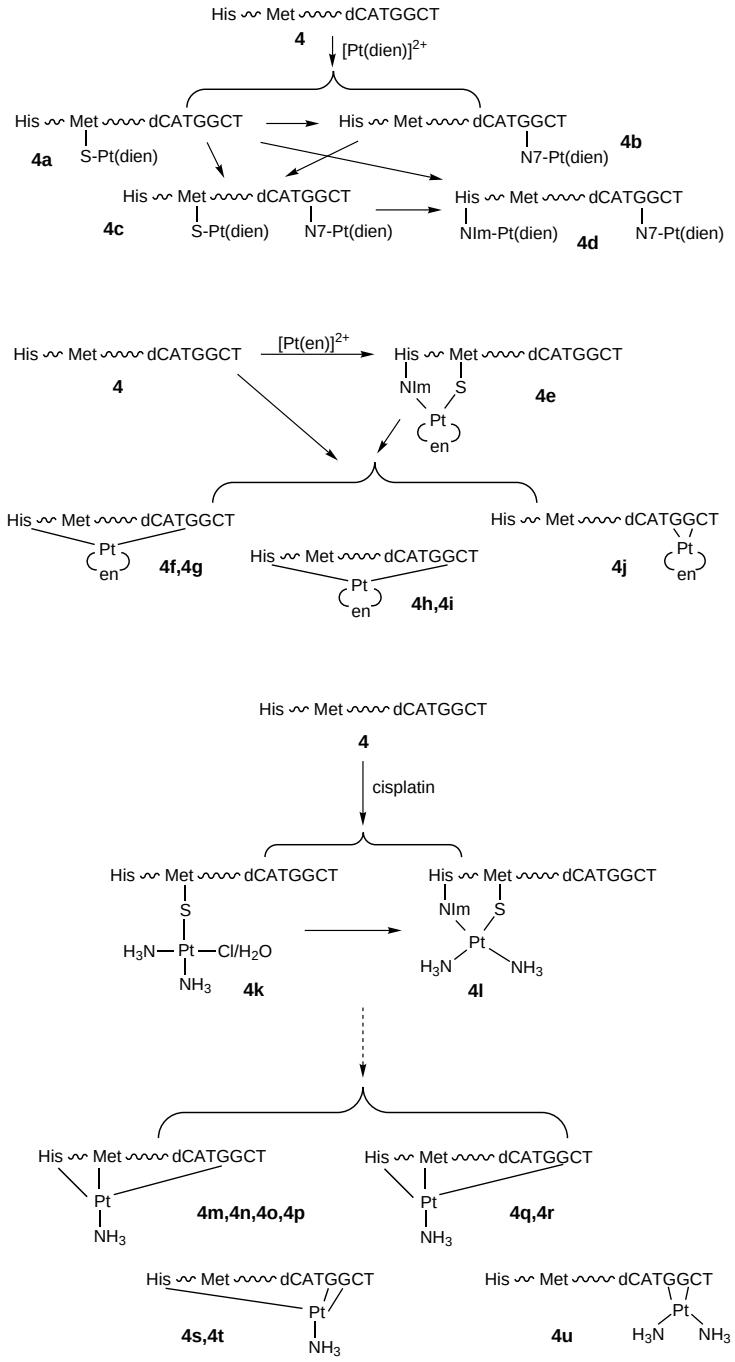

Scheme 4. Products formed in the reaction between the Pt^{II} complexes and hybrid **4** (Phac-L-Met-Gly-L-His-linker-p5′dCATGGCT).

(**3k**–**3n**, **4m**–**4r**). Only the formation of **4s** and **4t** (which were obtained in low amount) can be explained by a sulfur to nitrogen migration. Consequently, the hypothesis that cisplatin might be transported from proteins to DNA as a result of S to N7 migration should be reconsidered.

With respect to the use of $[Pt(en)]^{2+}$ as a model of cisplatin, reactions with the latter are much more complex than with the former. Only stable chelates are formed in the reaction with $[Pt(en)]^{2+}$, whilst cisplatin gives rise to the formation of monofunctional complexes, chelates and even bifunctional and trifunctional adducts; also the ammine *trans* to sulfur may be lost.

Loss of the ammine *trans* to sulfur in reactions with cisplatin is not at all unexpected,[12] but its consequences have to some extent been underestimated. Different reaction paths might account for the formation of Pt–S,NIm,N7 trifunctional complexes such as **3k**–**3n** or **4m**–**4t**. The ammine group may be lost from the monofunctional Pt–S adduct or from Pt–S,NIm or Pt–S,N7 complexes, and the Pt–N7 bond can be formed either by S to N7 migration or by introduction of the guanine ligand at the position previously occupied by ammine. Such processes taking place in a biological medium might give rise to platinum cross-linked protein–DNA trifunctional adducts whose biological significance is still unclear. It might be possible that trifunctional adducts are involved in one of the roles suggested for cisplatin, the hijacking of proteins away from their normal binding sites,[13] and may also account for some of the effects of the drug. Certainly only a small fraction of the products isolated from in vivo and in vitro experiments seems to correspond to DNA–Pt–protein complexes,[13] but no data are available on their repair efficiency, nor concerning their stability in cells or on the conditions used to isolate platinum-containing products from cell cultures.

We would also point out that for the structural analysis of the most complex adducts we have used, for the first time, information from mass spectrometric analysis after treatment with proteases or reaction with H_2O_2 to infer which platinum–amino-acid bonds had been established. Adducts with Pt–NIm linkages remained stable after digestion with either papaine or pronase, whereas the Phac-His-Gly fragment was removed from the complex when platinum was coordinated to the methionine side chain. Adducts with amino-acid–Pt(dien) linkages had slightly different behaviour; pronase cleaved Pt–S bonds in 30 min and, therefore, removed the entire peptide moiety. The NIm–Pt(dien) bond was cleaved after a 24 h treatment with pronase. A short reaction with nuclease S1 provided information on whether chelates with an

an amino-acid–metal–nucleobase cross-link had been formed.

Finally, assuming that the GG–cisplatin complex is responsible for the therapeutic effect, the results obtained upon reaction with **4** are consistent with loss of large amounts of the drug upon in vivo administration, since only a very small amount of the "therapeutic complex" **4u** was formed. Further studies with more complex hybrids and also in the presence of rescue agents[2] will be helpful both to get a deeper insight into their mode of action and to design more specific platinum-derived anticancer drugs.

Experimental Section

Reaction between the hybrids and platinum(II) complexes: Complexation reactions were carried out in H_2O at 37 °C. The required volume of a 10 mM aqueous solution of platinum(II) complex was added to the hybrid, previously liophylised in an eppendorf tube and dissolved in H_2O, so that the relative proportion hybrid/platinum(II) complex was 1:0.95. The solutions were 1.5 mM in hybrid in reactions with [Pt(dien)Cl]Cl and 1 mM in reactions with $[Pt(en)Cl_2]$ and cisplatin. $[Pt(en)Cl_2]$ and cisplatin were solubilised and aquatised by heating for 10–15 min at 90 °C, and the required amount of the resulting solution was immediately added to the solution of the hybrid. The pH of the reaction mixtures was checked and found to be slightly acidic (pH 4–6). No further pH adjustment was made except in one of the reactions of **2** with cisplatin, which was carried out at pH 8.

The evolution of the hybrid–[Pt^{II} complex] reaction (37 °C) was monitored by reversed-phase HPLC with on-line UV detection. The aliquots separated from the reaction mixture were kept frozen (after KCl addition in the case of the reactions with cisplatin) until they were analyzed. Reactions with ^{15}N-cisplatin were also monitored by [^{1}H,^{15}N]-HSQC NMR. ^{15}N-Cisplatin was prepared by reaction of K_2PtCl_4 with [99 % ^{15}N]-NH_4OAc following described procedures.[14] ^{1}H and [^{1}H,^{15}N]-HSQC NMR experiments were carried out at 500 MHz (Varian VXR or Bruker Advance DXR), except for the measurement of the ^3J or $^4J_{Pt,H}$ coupling constants which were made at 100 MHz (Bruker). These data allowed the platinum coordination site at the imidazole ring of **2f** and **2g** to be unequivocally established.

HPLC analysis of the reaction crudes was carried out on Nucleosil C18 columns (250 × 4 mm, 10 μm), at 1 mL min^{-1}, with aqueous ammonium acetate (0.01M) as solvent A and acetonitrile as solvent B, and one of the following linear gradients: a) from 15 to 30 % of B in 30 min, from 30 to 60 % of B in 30 min, and from 60 to 100 % of B in 1 min; b) from 5 to 25 % of B in 15 min and from 25 to 100 % of B in 30 min; c) from 5 to 25 % of B in 15 min and from 25 to 100 % of B in 20 min; d) from 5 to 25 % of B in 35 min and from 25 to 100 % of B in 1 min; e) from 5 to 25 % of B in 40 min and from 25 to 100 % of B in 1 min. Gradient a) was used for the analysis of the reaction **1** with $[Pt(dien)]^{2+}$; gradient b) to monitor the reactions of **1, 2** and **3** with $[Pt(en)]^{2+}$; gradient d) to monitor the reactions of **4** with $[Pt(dien)]^{2+}$ and $[Pt(en)]^{2+}$; gradient e) in the case **4** and cisplatin, and gradient c) in all the other cases.

Isolation of the platinum adducts: Platinum adducts were generally isolated after several HPLC runs by using analytical separation conditions, but a semipreparative Kromasil C18 column (250 × 10 mm, 10 μm, 3 mL min^{-1}) and the same gradient were used to isolate adducts from reactions of **2** with cisplatin, **4** with $[Pt(dien)]^{2+}$ and **4** with $[Pt(en)]^{2+}$.

t_R (min, analytical conditions): **1a** 15.3, **1b** 29.1, **1c** 24.1, **1d** 18.8, **1e** 21.0, **1f** 23.3, **1g** 14.3; **2a** 23.3, **2b** 23.1, **2c** 24.4, **2d** 19.0, **2e** 24.1, **2f** 21.6, **2g** 22.4; **3a** 23.0, **3b** 26.1, **3c** 24.5, **3d** 23.8, **3e** 26.0, **3f** 18.9, **3g** 20.7, **3h** 21.4, **3i** 22.8, **3j** 23.7, **3k**/**3l** 21.8/21.9 (these adducts were collected together), **3m** 23.2, **3n** 26.5; **4a** 17.1, **4b** 21.0, **4c** 19.7, **4d** 19.5, **4e** 17.6, **4f**/**4g** 17.1/18.5 (these adducts coeluted in the semipreparative purification conditions used and were characterised together), **4h** 15.5, **4i** 15.9, **4j** 23.9, **4k** 17.5, **4l** 16.8, **4m** 14.4, **4n** 14.9, **4o** 16.1, **4p** 16.6, **4q**/**4r** 18.1/18.4 (these adducts were collected together), **4s** 20.8, **4t** 21.3, **4u** 22.1.

Characterisation of the platinum adducts formed from 1, 2 and 3: All of the isolated products were characterised by either MALDI-TOF or electrospray mass spectrometry, with detection in the positive mode (some spectra were also obtained in the negative mode).

Ultraviolet spectra were recorded for all the products isolated from the reaction of **1**, **2** and **3** with the Pt^{II} complexes. Coordination to the N7 of guanine shifted the absorption maximum from 252 to 258 nm, with small differences in compounds **1g** (256 nm), **2f** (260 nm), and **3k** and **3l** (256 nm).

Stability to snake venom phosphodiesterase (**1b**, **1c**, **1e**–**1g**, **2a**, **2e**–**2g**, **3b**, **3e**, **3k**–**3n**) was an additional indication of a Pt–N7 bond (**1a**, **1d**, **3a**, **3c** and **3d** were degraded).

^{1}H NMR (500 MHz), in particular the chemical shifts of the methyl group of the methionine residue, of the H2 and H5 imidazole protons and of the guanine H8, was also used for the structural analysis of compounds **1a**–**1c**, **1g**, **2a**, **2e**–**2g**, **3b**, **3e** and **3k**–**3n**. Coordination to the Nπ in **2f** was inferred from the Pt–H J values of the imidazole protons (H2: $^3J = 20$ Hz, H5: $^4J = 8$ Hz); in the case of Pt–Nτ isomer **2g** the two coupling constants were similar (15–20 Hz). The $^{15}NH_3$ chemical shifts obtained from [^{1}H,^{15}N]-HSQC experiments showed the presence of ammine groups placed *trans* to either sulfur or nitrogen atoms (**1d**, **1f**, **1g**, **2e**, **2f**, **3f**, **3h**). This NMR experiment showed the absence of ammine *trans* to sulfur in the final products of the reaction mixtures of **1** and **3** with cisplatin. Invariability of the imidazole ^{1}H chemical shifts at different pH values indicated coordination of the metal to the histidine residue (**2f**, **2g**, **3n**).

If an m/z value 16 units higher than that of the parent compound was not found after treatment with H_2O_2, we inferred that platinum was linked to the sulfur of methionine (**3d**, **3e**, **3h**, **3i**).

Characterisation of the platinum adducts obtained from hybrid 4: Mass spectrometric data (MALDI-TOF, negative mode) were used for the structural analysis of all the platinated products obtained from the most complex hybrid, **4**. Structural information was also obtained from treatment with different enzymes (phosphodiesterases or proteases) or reaction with H_2O_2 followed by mass spectrometric analysis. [^{1}H,^{15}N]-HSQC NMR experiments provided evidence of the presence of ammine *trans* to sulfur at the beginning of the reaction with cisplatin (**4k** and **4l**).

Treatment with snake venom phosphodiesterase (30 min): indiscriminate cleavage of the oligonucleotide moiety indicated absence of coordination to any of the guanines (**4a**, **4e**), removal of the fragment pGpCpT denoted coordination to the 5′G nucleobase (**4b**–**4d**, **4f**, **4g**, **4m**–**4p**) and elimination of pCpT indicated coordination to the 3′G nucleobase (**4h**–**4j**, **4q**–**4u**).

Nuclease S1 treatment (10 min) of chelates with a peptide–Pt–oligonucleotide linkage (**4f**–**4i**, **4m**–**4t**) afforded, among others, a product with an m/z ratio 18 units higher than that of the parent compound; this corresponded to the cleavage of a single phosphodiester bond with the subsequent addition of one water molecule. The m/z value 18 units higher than that of the parent compound was not found when **4e**, **4j** and **4u** were submitted to digestion with nuclease S1.

Removal of the fragment Phac-His-Gly upon reaction with papaine (30 min) indicated no Pt–Im coordination (**4a**–**4c**, **4j**, **4k**, **4u**), whereas compounds with Pt–NIm bonds were stable to this enzyme (**4d**–**4i**, **4l**–**4t**).

Complexes with Pt–NIm bonds (**4d**–**4i**, **4m**–**4t**) were not degraded by a 30 min treatment with pronase, but a 24 h treatment was able to cleave the Pt(dien)–imidazole bond (**4d**). Pronase digestion yielded the linker-oligonucleotide fragment either when platinum was linked to the oligonucleotide (**4b**, **4j**, **4u**) or from compounds with S–Pt(dien) bonds (**4a**, **4c**).

No reaction with H_2O_2 confirmed coordination of platinum to sulfur in **4a**, **4c** and **4m**–**4r**; compounds with an m/z ratio 16 units higher, corresponding to oxidation of the "unprotected" thioether to sulfoxide, were obtained from **4s**, **4t** and **4u**.

Additional evidence of Pt–NIm,N7,N7 coordination in compounds **4s** and **4t** came from MS analysis after a 48 h treatment with calf spleen phosphodiesterase, after which time a fragment with a mass corresponding to [Phac-His-Gly-Met(O)-OH+pGpG+Pt(NH_3)] was found (thioether to sulfoxide oxidation took place under those conditions).

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