Synthesis, Biophysical Studies, and Antiproliferative Activity of Platinum(II) Complexes Having 1,2-Bis(aminomethyl)carbobicyclic Ligands

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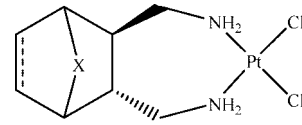
A selected chemical library of six platinum(II) complexes having 1,2-bis(aminomethyl)carbobicyclic ligands were synthesized after a rational design in order to evaluate their antiproliferative activity and the structure–activity relationships. The cytotoxicity studies were performed using cancer cell lines sensitive (A2780) and resistant (A2780R) to cisplatin. Excellent cytotoxicity was observed for most of complexes, which presented better resistance factors than cisplatin against the A2780R cell line. The interaction of these complexes with DNA, as the target biomolecule, was evaluated by several methods: DNA−platinum binding kinetics, changes in the DNA melting temperature, evaluation of the unwinding angle of supercoiled DNA, evaluation of the interstrand cross-links, and replication mapping. The kinetics of the interaction with glutathione was also investigated to better understand the resistant factors observed for the new complexes.

Introduction

In the search for new platinum anticancer drugs, great efforts were devoted to the design of complexes more efficient and less toxic than the reference drugs already in clinical use. For this purpose, the rational design of new molecules and the study of relevant structure–activity relationships (SAR[a])have been extended to families of new compounds having high structural diversity.[1]

Platinum(II) complexes having a diamino ligand with the two N-donors attached to a six-membered carbocycle (such as the 1,2-diaminocyclohexane (DACH) ligand present in oxaliplatin) appear to have interesting anticancer activity.[2,3] We have extended the investigation to the synthesis and biological evaluation of cisplatinum(II) complexes with ligands containing a carbobicyclic framework and two methylamino substituents in 1,2 positions in order to have a 1,4-diaminobutane-like structure (Figure 1). This type of molecule was designed to slightly increase the flexibility and the steric hindrance of the diamine carrier ligand with respect to corresponding complexes with DACH ligands. Moreover, the stereochemistry and lipophilicity of the new ligands were modulated to investigate how they can influence the interaction with DNA, as the target molecule, and the pharmacokinetics of the compounds. To this end, several types of bridges were inserted into the cyclohexane template. By coordination to platinum, the new ligands generate a seven-member platinacycle instead of the usual five- or six-member metallacycles. In the literature there are already a few examples of seven-member platinacycles having better antiproliferative activities than their smaller analogues.[4–6] At present, only chlorides have been used as labile ligands; however, their substitutions with other anionic ligands, such as carboxylates or dihydroxylates, are underway in our laboratory. The pharmacokinetic and the bioavailability of the complexes can depend very much on the nature of the leaving ligands.



X= O, CH_2 or CH_2CH_2

Figure 1. Basic structure of the platinum compounds studied in this work.

In the present work, a selected chemical library of six platinum(II) complexes having 1,2-bis(aminomethyl)carbobicyclic ligands have been synthesized and their antiproliferative activity and structure–activity relationships were investigated. These compounds were prepared after a rational design of the changes introduced in the carrier ligand (Figure 1). The cytotoxic activity of these new platinum complexes was evaluated against human ovarian cancer cell lines sensitive (A2780) and resistant (A2780R) to cisplatin. In addition, since DNA is considered the major pharmacological target of platinum antitumor drugs, the interaction of these complexes with DNA was also evaluated by using several biochemical and biophysical methods: DNA−platinum binding kinetics, changes in the DNA melting temperature, evaluation of the unwinding angle of supercoiled DNA, and evaluation of the interstrand cross-links and transcription mapping of the DNA adducts. The kinetics of the interaction with glutathione was also investigated and put in relation with the good resistant factors observed for the new platinum complexes.

Results and Discussion

Synthesis of Cisplatinum(II) Complexes. In order to obtain the desired compounds, six bicyclic diamines were synthesized by a Diels–Alder reaction[7] between fumaronitrile (as dienophile)

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[a]Abbreviations: CT, calf thymus; DPP, differential pulse polarography; FAAS, flameless atomic absorption spectrophotometry; GSH, glutathione; SAR, structure–activity relationship; t_m, melting temperature.

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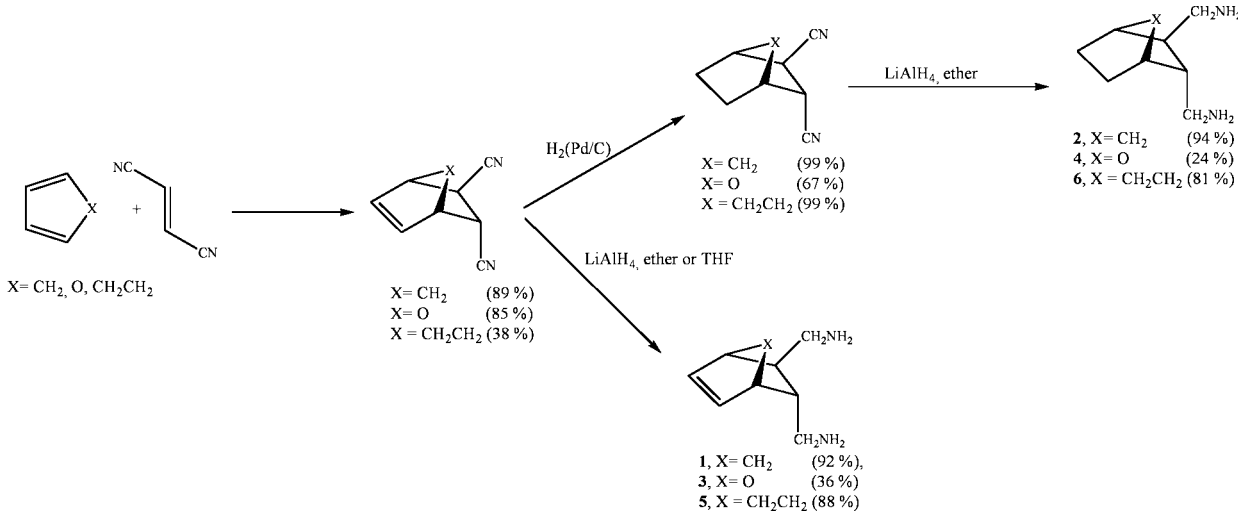

Figure 2. Organic synthesis of diamines **1–6**.

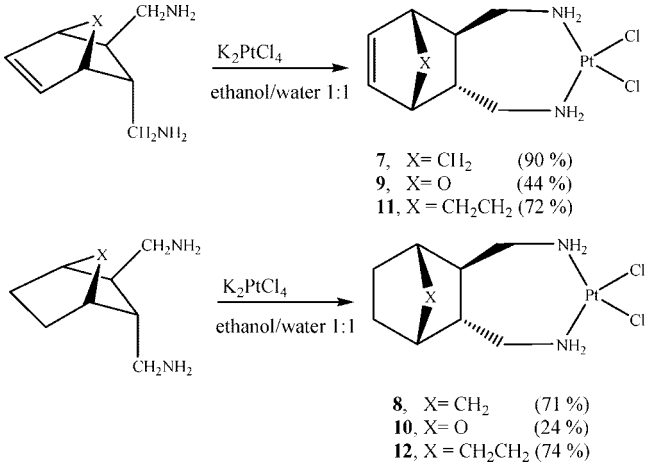

Figure 3. Formation of platinum(II) complexes from diamines **1**−**6**.

and an appropriate diene (such as furan, cyclopentadiene, or cyclohexadiene). In order to prepare the corresponding compounds without a double bond, the intermediate nitriles were hydrogenated in the presence of Pd/C catalyst. Nitriles were reduced with $LiAlH_4$ in diethyl ether (or THF in the case of compound **3**) to yield the corresponding diamines **1–6** (Figure 2). Finally, the diamines were reacted with K_2PtCl_4 in 1:1 ethanol/water to yield the corresponding cisplatinum(II) complexes (Figure 3).

In this synthetic pathway we observe a great difference in reactivity between the compounds with a hydrocarbon bridge (**1**, **2**, **5**, and **6**) and those with an oxygen bridge (**3** and **4**). The synthesis of the oxygen-bridged compounds was more difficult because of the formation of byproducts in nearly all synthetic steps and the high susceptibility of the cycloadduct to undergo a retro-Diels–Alder reaction regenerating the starting materials furan and fumaronitrile.

In the cases of ligands **5** and **6** the Diels–Alder reaction between cyclohexadiene and fumaronitrile was rather difficult and the reactivity was enhanced by addition of $BF_3 \cdot OEt_2$ in catalytic amounts.

The six diamines were coordinated to platinum, and except for the oxygen bridged diamines, the platinum complexes were obtained in medium to good yields (however, complexes **9** and **10** were obtained with yields between 20% and 50%).

Table 1. Kinetics of the Binding of **7**−**12** and Cisplatin to Calf-Thymus DNA in a Medium of 10 mM $NaClO_4$ at 37 °C Determined by Differential Pulse Polarographic Assay[a]

compd	$t_{50\%}$[b] (min)
7	73
8	91
9	83
10	64
11	110
12	192
cisplatin	120

[a] The concentration of DNA was 32 μg/mL and $r_i = 0.05$. [b] The times at which the binding reached 50%.

Biochemical and Biophysical Analysis of DNA Modifications by Platinum Complexes. DNA Binding. Solutions of double-helical calf thymus (CT) DNA at 0.1 mg/mL were incubated with **7**−**12** at an r_i of 0.05 in 10 mM $NaClO_4$ at 37 °C (r_i is defined as the molar ratio of added platinum complex to nucleotide phosphates at the onset of incubation with DNA). At various time intervals, an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography (DPP) for platinum not bound to DNA.[8] The amount of platinum bound to DNA, r_b (r_b is defined as the number of molecules of the platinum compound bound per nucleotide residue) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum present in the reaction. The amount of the platinum compounds bound to DNA increased with time, and after approximately 10 h all complexes tested were quantitatively bound. The times at which the binding reached 50% ($t_{50\%}$) in these binding reactions are shown in Table 1.

The values of $t_{50\%}$ for all compounds tested were comparable to that obtained for cisplatin.[9] Interestingly, the oxygen bridged compounds reacted more quickly than cisplatin. In the case of carbon bridged compounds, there is an interesting relationship between the values of $t_{50\%}$ and the ligand bulkiness. Thus, the compounds that have smaller bicyclic ligands (**7** versus **11** and **8** versus **12**) exhibit also smaller values of $t_{50\%}$.

If we compare the complexes on the basis of the presence or absence of a double bond (**7** versus **8** and **11** versus **12**), we observe significantly smaller values of $t_{50\%}$ for compounds carrying a double bond (especially in the case of **11** versus **12**). In the case of the oxygen-bridged compounds (**9** and **10**) the values of $t_{50\%}$ were, on the average, smaller than in the other four cases, indicating that the oxygen atom plays a role in

favoring the approach of the compound to the target DNA. Moreover, in this case the behavior of the compounds having or lacking a carbon−carbon π-system is reversed (smaller values of $t_{50\%}$ found for the compound lacking the π-system). It is possible to hypothesize that in the case of oxygen bridged compounds the approach to DNA is led, to some extent, by the oxygen atom and therefore the additional effect of a double bond could be negligible if not detrimental. In contrast, in the case of compounds lacking an oxygen atom, the approach to DNA could be favored, to some extent, by the π-electron system of the C═C double bond when present.

The binding experiments indicate that after 24 h of reaction time all molecules of the platinum complex are bound to DNA, therefore making it possible to prepare easily and precisely DNA samples modified at a preselected value of r_b.

Sequence Preference of DNA Adducts. This procedure involved the extension by TaKaRa Taq DNA polymerase (which exhibits extreme thermostability) at the 3′ end of a 5′ end radioactively labeled primer up to the site of platination of the template strand of pSP73 plasmid (2464 base pairs). The products of the linear amplification were then examined on DNA sequencing gels, and the sequence specificity of the platinum adduct formation was determined to the exact base pair. In vitro DNA synthesis on double-stranded templates, containing the adducts of **7**−**12**, generated a population of DNA fragments, indicating that these adducts terminate duplex synthesis (Figure 4A, lanes 1–6). Sequence analysis of the termination sites produced by **7**−**12** (Figure 4) shows that these complexes exhibit identical sequence dependence of the inhibition and also are clearly identical to that exhibited by cisplatin (Figure 4A, lane 7). They correspond to guanines (G) and to a considerably lesser extent to adenines (A). These G and A sites were mostly contained in GG or AG sequences, which are also the preferential DNA binding sites for cisplatin. Taken together, the results of the mapping experiments suggest that the base sequence selectivity of cisplatin and **7**–**12** is similar so that the major adducts formed on DNA by **7**–**12** and cisplatin are also identical, presumably 1,2-GG or AG intrastrand cross-links.

Interstrand Cross-Linking. The amounts of interstrand cross-links formed by **7**−**12** in linear DNA were measured in pSP73 plasmid, which was first linearized by EcoRI (EcoRI cuts only once within pSP73 plasmid) and subsequently modified by **7**–**12** at $r_b = 0.001$. The samples were analyzed for the interstrand cross-links by agarose gel electrophoresis under denaturing conditions. An electrophoretic method for precise and quantitative determination of interstrand cross-linking by platinum complexes in DNA was described previously.[10–12] Upon electrophoresis under denaturing conditions, 3′-end labeled strands of linearized pSP73 plasmid containing no interstrand cross-links migrate as a 2464-base single strand whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The band corresponding to more slowly migrating interstrand-cross-linked fragments was seen in all experiments where platinum complexes **7**–**12** were used to modify linearized DNA (Figure 5). The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA. The frequency of interstrand cross-links (the amount of interstrand cross-links per one molecule of **7**–**12** bound to DNA) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size[10–12] (Table 2). Compounds **7**–**12** showed a similar or slightly lower cross-linking efficiency (2–6%) as parent cisplatin (5–6%).[11]

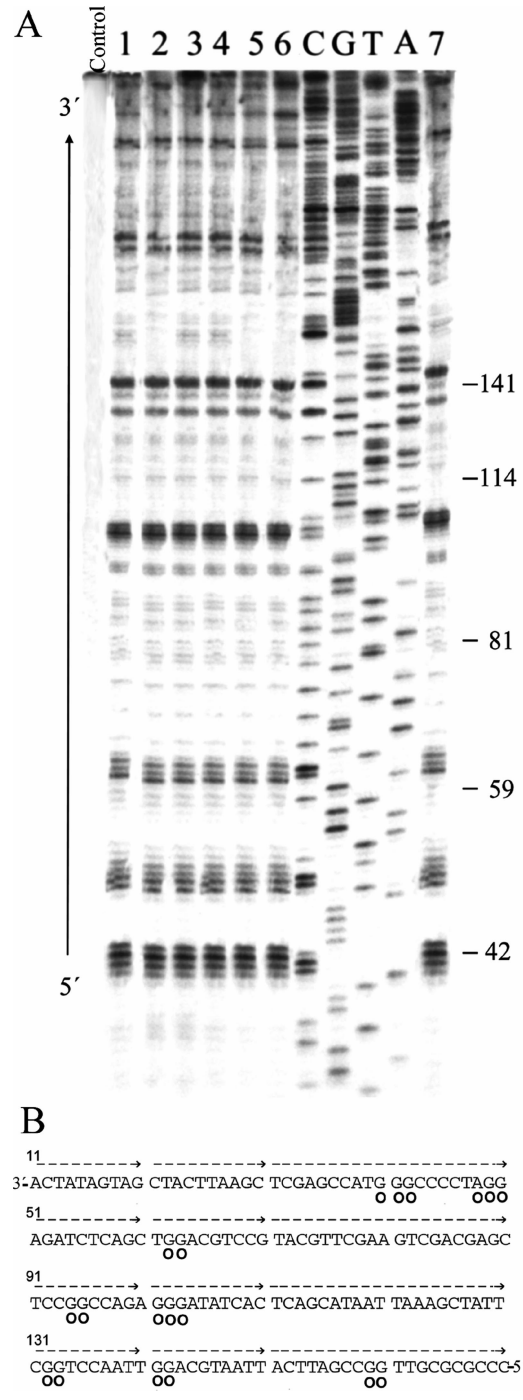

Figure 4. (A) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by TaKaRa Taq DNA polymerase on the pSP73 plasmid DNA linearized by HpaI restriction enzyme and subsequently modified by platinum complexes. The gel contained the linear amplification products of control, nonplatinated DNA, and DNA treated with **7**−**12** or with cisplatin at $r_b = 0.006$. Lanes are as follows: (control) unmodified template; (lanes 1–6) DNA modified by **7**–**12**, respectively; (lane 7) DNA modified by cisplatin; (C, G, T, A) chain-terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand). The numbers correspond to the nucleotide sequence numbering of part B. (B) Schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of **7**. The arrows indicate the direction of the synthesis. Circles indicate major stop signals from part A, lane 1. The numbering of the nucleotides in this scheme corresponds to the numbering of the nucleotides in the pSP73 nucleotide sequence map.

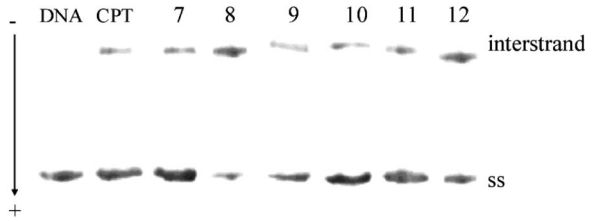

Figure 5. Formation of interstrand cross-links by platinum complexes in linearized pSP73 plasmid. Autoradiogram of denaturing 1% agarose gels of linearized DNA that was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). Plasmid linearized by EcoRI was incubated for 48 h with platinum complexes at r_b values of 0 (lane DNA) or 0.001 (lane CPT, **7**–**12**). Lanes are as follows: (CPT) DNA modified by cisplatin; (lanes **7**–**12**) DNA modified by **7**–**12**, respectively.

Table 2. Frequency of Interstrand Cross-Links (% ICL/Pt) Formed by **7**−**12** and Cisplatin in Linearized pSP73 Plasmid (2464 Base Pairs), $r_b = 0.001$[a]

compd	% ICL/Pt
7	3.8
8	3.6
9	3.2
10	4.3
11	2.1
12	5.6
cisplatin	4.8

[a] Linearized plasmid was incubated with the platinum complex for 48 h in 10 mM $NaClO_4$ at 37 °C.

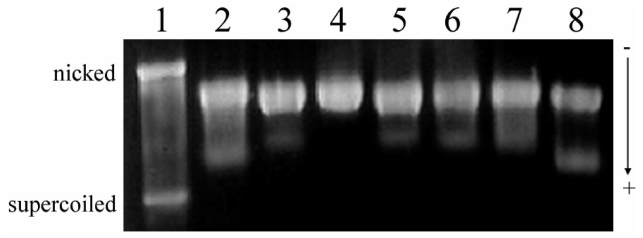

Figure 6. Unwinding of supercoiled pSP73 plasmid DNA by compound **12**. The plasmid was incubated with the platinum complex for 48 h at 37 °C. Lanes are as follows: (lane 1) control, nonmodified DNA ($r_b = 0$); (lane 2) $r_b = 0.02$; (lane 3) $r_b = 0.03$; (lane 4) $r_b = 0.04$; (lane 5) $r_b = 0.05$; (lane 6) $r_b = 0.06$; (lane 7) $r_b = 0.07$; (lane 8) $r_b = 0.08$. The top bands correspond to a form of nicked plasmid, and the bottom bands correspond to the closed, negatively supercoiled plasmid.

Unwinding Induced in Supercoiled Plasmid DNA. Electrophoresis in native agarose gel was used to quantify the unwinding induced in pSP73 plasmid by the platinum complexes **7**–**12** by monitoring the degree of supercoiling (Figure 6). A compound that unwinds the DNA duplex reduces the number of supercoils so that the superhelical density of closed circular DNA decreases. This decrease of superhelical density induced by binding of unwinding agents causes a decrease in the rate of migration through the agarose gel, from which the degree of unwinding can be quantified.[13] Figure 6 shows electrophoresis gel results in which increasing amounts of compound **12** were bound to a mixture of relaxed and supercoiled pSP73 DNA. Interestingly, the platinum complex accelerates the mobility of the relaxed form similarly to what is observed in the case of cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix. The unwinding angle is given by $\Phi = -18\sigma/[r_b(c)]$ where σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and relaxed forms comigrate. Under the present experimental conditions, σ was calculated to be −0.036 on the basis of the data relative to cisplatin for which the $r_b(c)$ was determined in this study and Φ was assumed to be 13°.[13] The value of $r_b(c)$ at which the supercoiled and relaxed forms modified by **7**–**12** comigrate and the corresponding unwinding angles are summarized in Table 3.

Table 3. Unwinding of Supercoiled pSP73 DNA by **7**−**12** and Cisplatin[a]

compd	$r_b(c)$	unwinding angle[b] (deg)
7	0.065	10 ± 2
8	0.055	12 ± 2
9	0.065	10 ± 2
10	0.065	10 ± 2
11	0.045	14 ± 2
12	0.04	16 ± 2
cisplatin		13°[6]

[a] Plasmid was incubated with the platinum complex for 48 h in 10 mM $NaClO_4$ at 37 °C. [b] The unwinding angle was calculated as described in the text.

In these assays we observed a relationship between unwinding angle and steric bulkiness similar to that observed in the platinum−DNA binding studies (Table 1). Therefore, ligands that have a smaller size (**7** versus **11** and **8** versus **12**) produced smaller values of unwinding angle. Similarly, ligands containing a C═C π-system (**7** and **11**) produced smaller unwinding angles with respect to compounds lacking the π-system (**8** and **12**). A different trend was observed for the oxygen-bridged complexes. These compounds (**9** and **10**) gave, on the average, smaller unwinding angles than the other four compounds, and the presence or absence of a C═C double bond had practically no effect. Apparently the oxygen bridge can produce some kind of additional interaction with DNA, which minimizes unwinding and offsets any effect of a C═C π-system. In fact, the unwinding angle was very close to that of cisplatin for all compounds with the only exception being compound **12**, which carries the bulkiest ligand, lacks a π-system, and produces an unwinding angle larger than that of cisplatin. From the previous observations we can conclude that in this series of bicyclic ligands the unwinding angle is modulated by two factors:

(a) One factor is the presence of an oxygen bridge or an electron π-system. It causes a lowering of the unwinding angle (and we have previously observed that it also increases the rate of DNA binding). It is reasonable to propose that the oxygen atom or the electron π-system can produce some additional interactions (electrostatic interactions, hydrogen bonds, or π-stacking) that can lead to a better fitting of the complex into the supercoiled DNA structure.

(b) Another factor is the steric bulk of the ligands. As the size of the ligand increased, there is also an increase of Φ (and also a reduction in the rate of interaction with DNA). This phenomenon could be interpreted as a consequence of the more intense distortion produced by bulkier compounds when they interact with DNA.

DNA Melting. CT DNA was modified by **7**–**12** to $r_b = 0.05$ in 10 mM $NaClO_4$. Salt concentration was then further adjusted by addition of $NaClO_4$ to produce the values of 0.01 or 0.2 M. The effect on DNA melting temperature (t_m) is dependent on both the amount of platinum bound and the salt concentration.[14]

All compounds showed a similar behavior. At low ionic strength there was only a small decrease of t_m or no change at all with respect to free DNA (the observed decrease of t_m was from 0 to 2.7 °C; see Table 4). However, at high ionic strength, the decrease of t_m was greater because of the lower stabilization imposed on DNA by the positive charge of platinum (the observed decrease of t_m was between 3.4 and 8 °C). Under

Table 4. t_m Values for Calf-Thymus DNA Modified by **7**−**12** at r_b = 0.05

compd	t_m (°C) (low ionic strength[a])	t_m (°C) (high ionic strength[b])
7	64.4	82.5
8	67.3	84.0
9	65.7	79.0
10	66.5	83.6
11	66.3	81.6
12	66.8	83.3
noPt	67.1	87.0

[a] 0.01 M $NaClO_4$ with 1 mM Tris-HCl/0.1 mM EDTA, pH 7.4. [b] 0.2 M $NaClO_4$ with 1 mM Tris-HCl/0.1 mM EDTA, pH 7.4.

Table 5. Half-Times ($t_{1/2}$) of Reaction of the Reduced Form of Glutathione with **7**−**12** and Cisplatin[a]

compd	$t_{1/2}$ (min)
7	333
8	887
9	
10	61
11	835
12	131
cisplatin	22

[a] The platinum compounds at 23 μM were mixed with 12 mM glutathione at 37 °C in a medium of 0.1 M Tris-HCl, pH 7.4, plus 5 mM NaCl. For other details, see the text.

analogous conditions cisplatin produces a t_m decrease of 2.5 °C at low ionic strength and a decrease of 4.1 °C at high ionic strength.[14] In aggregate, the melting behavior of DNA modified by compounds **7**−**12** is very similar to that of DNA modified by cisplatin, which is consistent with the hypothesis that the DNA modifications caused by these complexes are similar to those caused by cisplatin.

Reactions with Glutathione. Platinum(II) compounds have a strong thermodynamic preference for binding to S-donor ligands. Hence, before antitumor platinum(II) drugs reach DNA in the nucleus of tumor cells or even after they bind to DNA, they may interact with various compounds including sulfur-containing molecules. These interactions are generally believed to play a role in mechanisms underlying tumor resistance to platinum compounds, their inactivation, and the side effects. Therefore, the interest in the interactions of platinum antitumor drugs with sulfur-containing molecules of biological significance has increased markedly.

An example of endogenous thiols to which platinum(II) complexes bind when they are administered intravenously or after they enter the cell is glutathione (GSH). In the present work, we investigated using UV absorption spectrophotometry[15] the reaction of GSH with **7**–**12**. Thus, **7**–**12** at 23 μM were mixed with 12 mM GSH (this concentration of GSH represents a physiologically relevant concentration) at 37 °C in the medium of 0.1 M Tris-HCl, pH 7.4, and 5 mM NaCl. The half-times of these reactions are shown in Table 5. There are significant differences among the compounds, but all compounds bound GSH with a slower rate than cisplatin. Interestingly, compound **9**, containing an oxygen bridge and a C═C π-system, does not cause an increase in absorption with respect to the blank (a solution of GSH). Additional work is needed to interpret this unexpected observation.

These results are rather important because they are consistent with the better resistant factors observed for the new compounds in the antiproliferative studies against resistant cancer cell lines (see below). This is worth noting because one of the mechanisms of acquired resistance of cancer cells is the enhanced drug inactivation fostered by greater GSH and metallothionein contents.[16]

Table 6. Biological Activity of Compounds **7**–**12** Tested against Cisplatin Sensitive (A2780) and Resistant (A2780R) Cell Lines[a]

compd	IC_{50} (μM) A2780	IC_{50} (μM) A2780R	resistance factor	cytotoxicity relative to cisplatin[b]
7	1.9 ± 0.9	9.2 ± 3	4.7	0.16
8	0.3 ± 0.1	2.3 ± 0.2	8.7	1.00
9	0.07 ± 0.002	0.4 ± 0.007	5.6	4.28
10	1.7 ± 0.01	19.7 ± 0.01	11.6	0.18
11	0.12 ± 0.05	0.48 ± 0.2	4.2	2.50
12	0.07 ± 0.005	0.2 ± 0.05	3.5	4.28
cisplatin	0.3 ± 0.06	4.5 ± 0.7	17.3	1.00

[a] The cytotoxicity of evaluated complexes relative to cisplatin was estimated as IC_{50}(cisplatin)/IC_{50}(complexes). [b] Cytotoxicity relative to cisplatin: IC_{50}(cisplatin)/IC_{50}(compound).

Assays of Cytotoxicity. The antiproliferative activity of compounds **7**−**12** was studied against A2780 and A2780cisR human ovarian cancer cell lines sensitive and resistant to cisplatin, respectively. The work with cisplatin-resistant cancer cell lines was carried out because of the fact that cell resistance, either intrinsic or acquired, is one of the main limitations to the clinical use of cisplatin. The capability of a new compound to overcome cisplatin resistance is expressed by the resistance factor (RF = (IC_{50} resistant cell lines)/(IC_{50} sensitive cell lines)); the lower the RF value, the better is the drug. Usually, acquired resistance is accompanied by a decrease in cellular transport, increase in DNA repair, and increase in glutathione level.[16–18] The IC_{50} values (concentrations that cause 50% decrease of the cellular growth) for 96 h of incubation time are quoted in Table 6.

We observe that compounds **9**, **11**, and **12** show much better biological activity than cisplatin. In fact, **9** and **12** are 4.28 times more active than cisplatin and **11** is 2.5 times more active. Compound **8** has activity similar to that of cisplatin, while complexes **7** and **10** are less active. It is worth noting that all compounds present lower resistance factors than cisplatin when they were tested against the cisplatin resistant cell line, showing that this family of complexes is able to partially overcome the cisplatin resistance developed by the A2780R cancer cell line. This important finding could be related, as mentioned before, to a lower drug inactivation by glutathione and other sulfur-containing platinophiles. Some SAR trends between the bicyclic residue of the ligands and the differences in biological activity can be established. Thus, for the carbon-bridged complexes (**7**, **8**, **11**, and **12**), the presence of a C═C π-system appears to produce a decrease in biological activity with respect to the complexes lacking such a π-system. Moreover, it is also found that an increase in steric bulkiness also produces an increase in biological activity (decrease in IC_{50} value) so that compounds with an ethylene bridge (**11**, **12**) afford better results than those with a methylene bridge (**7**, **8**). In the case of **11** and **12**, an increase in ligand lipophilicity could be the factor responsible for their higher cytotoxicity. In the case of complexes with an oxygen bridge (**9**, **10**), the presence of a C═C π-system (complex **9**) causes a relevant increase in biological activity with respect to the compound lacking such a π-system (complex **10**). Once more, the presence of an olefin π-system appears to have an opposite effect in the ligands containing carbon bridges with respect to those containing an oxygen bridge.

Conclusion

A series of platinum compounds with promising biological activities were synthesized by a two- or three-step synthetic pathway. Four of these compounds showed better antiproliferative activity than cisplatin (up to 4.28 times more active than

the clinically relevant drug). It is worth noting that all compounds exhibit lower resistance factors than cisplatin when tested against cisplatin resistant cell lines, showing that this family of complexes is able to partially overcome the cisplatin resistance developed by the A2780R cancer cell line. Moreover, the compounds that showed better in vitro biological activities and lower resistant factors were also the compounds reacting more slowly with glutathione. Therefore, it is possible to hypothesize that our compounds are able to overcome resistance stemming from drug inactivation by glutathione and other sulfur-containing platinophiles. Some SARs between the bicyclic ligand moieties and the complex biological activities appear to involve the steric bulkiness, the presence of an olefinic π-system, and the presence of a carbon/oxygen bridge. The biophysical studies showed that this family of compounds behaves like cisplatin as far as interaction with DNA is concerned.

Because of the interesting properties and the promising potential as anticancer agents of this series of compounds, further studies will be carried out to optimize these leads on the basis of the preliminary SAR results to improve their efficiency, selectivity, and pharmacokinetic properties. Further studies on the resistance mechanisms will also be carried out.

Experimental Section

Chemical Synthesis of the Platinum(II) Complexes. Materials and Methods. NMR spectra were recorded on a Bruker 500 spectrometer using (DMSO-d_6) as solvent. Infrared spectra were recorded by a Nicolet 510 FT-IR spectrophotometer as films on NaCl crystal plates in the case of oils or as thin plates of the analyte dispersed in anhydrous KBr in the case of solids. Fast atom bombardment (FAB) mass spectra were obtained by using a 3-nitrobenzyl alcohol (NBA) matrix and dimethyl sulfoxide (DMSO) as solvent. Melting points were determined with an Electrothermal apparatus and are uncorrected. Elemental analyses (combustion analysis) were obtained with a Carlo Erba EA 1108 apparatus. For the synthesis and characterization of the diamine ligands **1**−**6**, see the Supporting Information.

Synthetic Methodology for the Preparation of the Platinum Compounds 7–12 Starting from Ligands 1–6. In a typical experiment a solution of **1** (81 mg, 0.53 mmol) in ethanol (5 mL) was added to a solution of K_2PtCl_4 (220 mg, 0.53 mmol) in water (5 mL). The reaction mixture was stirred in the dark for 24 h, and then the precipitate was separated from the mother solution by filtration through a sintered-glass plate (10–16 μm). The yellow precipitate was washed with water, ethanol, acetone, and dichloromethane and dried under vacuum to obtain 200 mg of product **7** (0.48 mmol, yield = 90%).

***cis*-{[(3-Aminomethylbicyclo[2.2.1]hept-5-en-2-yl)methylamine]dichlorido}platinum(II) (7).** Yellow solid. Mp = 240–242 °C. IR (KBr) 3440, 3250, 3210, 3120 (N−H, st), 3075, 2957, 2873, 1601, 1454 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.28–1.34 (m, 2H), 1.44 (d, J = 8.5 Hz, 1H), 1.50 (dd, J_1 = 8.5, 22.1 Hz, 1H), 2.55 (d, J = 4 Hz, 2H), 2.71–2.77 (m, 1H), 2.81 (s, 2H), 2.90 (d, J = 9.3 Hz, 1H), 4.92–4.97 (m, NH), 4.99–5.09 (m, 2NH), 5.14–5.21 (m, NH), 5.97 (m, 1H), 6.34 (dd, J = 3.0, 5.5 Hz, 1H) ppm. MS (MALDI-TOF) m/z 345.7 (M − 2Cl − H). Anal. ($C_9H_{16}N_2Cl_2Pt$) C, H, N.

***cis*-{[(3-Aminomethylbicyclo[2.2.1]hept-2-yl)methylamine]dichlorido}platinum(II) (8).** Yellow solid. Yield = 71%. Mp = 179–182 °C. IR (KBr) 3432, 3255, 3222, 3125 (N−H, st), 2948, 2875, 1603, 1452, 1383 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.13 (d, J = 9.5 Hz, 2H), 1.21–1.27 (m, 2H), 1.37–1.41 (m, 2H), 1.48–1.54 (m, 2H), 2.01–2.08 (m, 1H), 2.11–2.17 (m, 1H), 2.22–2.31 (m, 2H), 2.62–2.74 (m, 2H), 4.82 (s, NH), 4.96 (s, NH), 5.05 (s, NH) ppm. MS (MALDI-TOF) m/z 414 (M − 7H), 385 (M − Cl), 348 (M − 2Cl − H), 345 (M − 2Cl − 4H). Anal. ($C_9H_{18}N_2Cl_2Pt$) C, H, N.

***cis*-{[(3-Aminomethyl-7-oxabicyclo[2.2.1]hept-5-en-2-yl)methylamine]dichlorido}platinum(II) (9).** Yellow solid. Yield = 44%. Mp = 230–232 °C. IR (KBr) 3459, 3226, 3150 (N−H, st), 2950, 2886, 1593, 1456 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.59–1.67 (m, 1H), 2.07–2.16 (m, 1H), 2.24–2.38 (m, 1H) 2.4–2.45 (m, 1H), 2.75–2.83 (m, 1H), 2.91–2.97 (m, 1H), 4.66 (s, 1H), 4.92 (d, J = 4 Hz, 1H), 5.04 (m, 2NH), 5.15 (s, NH), 5.50 (s, NH), 6.24 (m, 1H), 6.61 (dd, J = 1.5, 5.5 Hz, 1H) ppm. MS [FAB (+)] m/z 502 (M − 2Cl + NBA), 537 (M − Cl + NBA). Anal. ($C_8H_{14}ON_2Cl_2Pt$) C, H, N.

***cis*-{[(3-Aminomethyl-7-oxabicyclo[2.2.1]hept-2-yl)methylamine]dichlorido}platinum(II) (10).** Yellow solid. Yield = 24%. Mp = 248–250 °C. IR (KBr) 3492, 3270, 3226, 3150 (N−H, st), 2969, 2900, 1589, 1456 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.42–1.62 (m, 4H), 1.9–2.07 (m, 1H), 2.19–2.27 (m, 1H), 2.28–2.34 (m, 1H), 2.57–2.63 (m, 1H), 2.68−2.75 (m, 1H), 2.85 (m, 1H), 4.2 (d, J = 4.5 Hz, 1H), 4.5 (t, J = 4.7 Hz, 1H), 4.85 (s, NH), 4.95–5 (m, NH), 5.12 (s, 2NH) ppm. MS (MALDI-TOF) m/z 351 (M − 2Cl − H), 386 (M − Cl). Anal. ($C_8H_{16}ON_2Cl_2Pt$) C, H, N.

***cis*-{[(3-Aminomethylbicyclo[2.2.2]oct-5-en-2-yl)methylamine]dichlorido}platinum(II) (11).** Yellow solid. Yield = 72%. Mp = 245–247 °C. IR (KBr) 3444, 3220, 3210 (N−H, st), 3130, 3050, 2940, 2873, 1597, 1456 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.99–1.08 (m, 1H), 1.16–1.26 (m, 1H), 1.48–1.61 (m, 2H), 1.77–1.83 (m, 1H), 1.85–1.94 (m, 1H), 2.34–2.43 (m, 3H), 2.61 (dd, J = 4.0, 8.0 Hz, 1H), 2.79–2.95 (m, 2H), 4.90–5.00 (m, NH), 5.04–5.18 (s, NH), 5.38–5.46 (m, NH), 5.62–5.82 (m, NH), 6.11 (dd, J = 1.0, 7.0 Hz, 1H), 6.38 (dd, J = 1.0, 7.0 Hz, 1H) ppm. MS (MALDI-TOF) m/z 359.9 (M − 2Cl − H). Anal. ($C_{10}H_{18}N_2Cl_2Pt$) C, H, N.

***cis*-{[(3-Aminomethylbicyclo[2.2.2]oct-2-yl)methylamine]dichlorido}platinum(II) (12).** Yellow solid. Yield = 74%. Mp = 185–187 °C. IR (KBr) 3444, 3270, 3222 (N−H, st), 3125, 2934, 2880, 1600, 1456 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.32–1.51 (m, 12H), 2.36–2.46 (m, 2H), 2.52–2.58 (m, 2H), 4.98 (s, 2NH), 5.16 (s, 2NH) ppm. MS (MALDI-TOF) m/z 355.9 (M − 2Cl − 7H). Anal. ($C_{10}H_{20}N_2Cl_2Pt$) C, H, N.

Biochemical and Biophysical Assays. Starting Materials. Stock solutions of platinum compounds for the biophysical and biochemical studies were prepared at the concentration of 5×10^{-4} M in 10 mM $NaClO_4$ and stored at 4 °C in the dark. The concentrations of platinum in the stock solutions were determined by flameless atomic absorption spectrophotometry (FAAS). CT DNA (42% G + C, mean molecular mass of about 2×10^7) was prepared and characterized as described previously.[19,20] pSP73 plasmid (superhelical density σ = 0.036) was isolated according to standard procedures. Restriction endonucleases Eco*R*I, HpaI, and T4 polynucleotide kinase were purchased from New England Biolabs. Klenow fragment from DNA polymerase I (wild type) was obtained from Takara (Japan). Acrylamide, bis(acrylamide), and ethidium bromide were obtained from Merck KgaA (Darmstadt, Germany). GSH was purchased from Sigma (Prague). Agarose was from FMC BioProducts (Rockland, ME). Radioactive products were from MP Biomedicals, (Irvine, CA).

Platination Reactions. DNA was incubated with platinum complex in 10 mM $NaClO_4$ at 37 °C for 48 h in the dark if not otherwise stated. The number of molecules of the platinum compound bound (coordinated) per nucleotide residue (r_b values) was determined by DPP[8] or FAAS. For other details, see the text.

Mapping of DNA Adducts. The linear fragment of pSP73 DNA was obtained as previously described.[11,21] A 10 μg amount of pSP73 was treated with HpaI to obtain linear plasmid (HpaI cuts only once within this plasmid). After deproteinization by phenol/chloroform, the modification of this fragment by the platinum complex was carried out in 10 mM $NaClO_4$ for 48 h at 37 °C to obtain r_b = 0.006. TaKaRa Taq cycle sequencing kit with TaKaRa Taq DNA polymerase was used along with the protocol for thermal cycle DNA sequencing with 5′ end-labeled primer recommended by the manufacturer with small modifications.[22]

DNA Interstrand Cross-Linking. Platinum complexes were incubated for 48 h with 1 μg of pSP73 DNA linearized by EcoRI

to reach $r_b = 0.001$. The linear DNA was first 3′-end labeled by means of Klenow fragment from DNA polymerase I in the presence of [α-^{32}P]dATP. The platinated samples were precipitated by ethanol and analyzed for DNA interstrand cross-links by previously published procedures.[10,11] After the platination, the samples were precipitated by ethanol and the pellet was dissolved in 18 μL of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose and 0.04% bromophenol blue. The amount of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified by means of a bioimaging analyzer.

DNA Melting. The melting curves of CT DNA were recorded by measuring the absorbance at 260 nm. The melting curves of unplatinated or platinated DNA were recorded after Tris-HCl/EDTA buffer and $NaClO_4$ were added so that the resulting media contained 0.01 or 0.2 M $NaClO_4$ with 1 mM Tris-HCl/0.1 mM EDTA, pH 7.4. The value of t_m was determined as the temperature corresponding to a maximum on the first-derivation profile of the melting curves. The t_m values could be thus determined with an accuracy of ±0.3 °C.

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay.[13] The unwinding angle Φ, induced per platinum−DNA adduct, was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to the relaxed form of the plasmid was attained. Samples of plasmid DNA were incubated with platinum complexes at 37 °C in the dark for 48 h. All samples were precipitated by ethanol and redissolved in the TAE (Tris-acetate/EDTA) buffer. An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer and the voltage set at 30 V. The gels were then stained with ethidium bromide, followed by photography with a transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

Reactions with Reduced Form of Glutathione. Reactions of GSH with platinum complexes were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH exactly as described in the previous work;[15,23] the absorbance at 260 nm reflects the presence of platinum−sulfur and disulfide bonds. The platinum compounds were mixed with GSH at 37 °C in a medium of 0.1 M Tris-HCl, pH 7.4, plus 5 mM NaCl, in the dark under nitrogen atmosphere. Reactions were initiated by mixing the platinum complex with the buffer followed by immediate addition of GSH.

Other Physical Methods. Absorption spectra were measured with a Varian Cary 4000 UV–vis spectrophotometer equipped with a thermoelectrically controlled cell holder and quartz cells with a path length of 1 cm. FAAS measurements were carried out with a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For FAAS analysis DNA was precipitated with ethanol and dissolved in 0.1 M HCl. The gels were visualized by using the BAS 2500 Fujifilm bioimaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software (Raytest, Germany).

Cytotoxicity Studies. The growth inhibitory effect of compounds under investigation was evaluated on the A2780/A2780cisR pair of human ovarian cancer cell lines (parent line from an untreated patient and derived cisplatin-resistant line). Tumour cells were kindly supplied by Dr. L. Kelland (The Institute of Cancer Research, Surrey, U.K.). A2780 and A2780cisR cells were maintained at 37 °C in a 10% CO_2 humidified air in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 2.5 μg/mL amphoterycin B, and 50 μg/mL gentamicin. All culture media and reagents were from Euroclone (Paignton, U.K.). The growth inhibitory effect of platinum compounds was evaluated by using the sulforhodamine-B (SRB) assay. Briefly, cells were seeded into 96-well microtiter plates in 100 μL of the appropriate culture medium at a plating density of 10 000 cells/well. After seeding, microtiter plates were incubated at 37 °C for 24 h prior to addition of the compounds. After 24 h, several samples of each cell line were fixed in situ with cold trichloroacetic acid (TCA) to represent a measurement of the cell population at the time of compound addition. Experimental compounds were freshly dissolved in culture medium and stepwise diluted to the desired final concentrations, following the addition of different compound concentrations to quadruplicate wells. The plates were further incubated at 37 °C for 96 h. Cells were fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10%) and incubated for 1 h at 4 °C. The supernatant was discarded, and the plates were washed four times with tap water and air-dried. SRB solution (100 μL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 30 min at room temperature. After the samples were stained, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was then dissolved in 10 mM TRIZMA [tris(hydroxymethyl)aminomethane] base and the absorbance was read on an automatic plate reader at 515 nm. The compound concentration able to inhibit cell growth by 50% ($IC_{50} \pm SD$) was then calculated from semilogarithmic dose-response plots obtained from three separate experiments.

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Supporting Information Available: Synthesis and characterization data of diamine ligands **1**−**6** and elemental analysis (combustion analysis) data for complexes **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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