Combined Effect of Platination and Intercalation upon DNA Binding of Novel Cytotoxic Pt−Bis(naphthalimide) Complexes

José M. Pérez,[†] Isabel López-Solera,[†] Eva I. Montero,[†] Miguel F. Braña,[‡] Carlos Alonso,[§] Simon P. Robinson,[||] and Carmen Navarro-Ranninger*,[†]

Departamento de Química Inorgánica, Universidad Autónoma de Madrid, 28049 Madrid, Spain, Universidad San Pablo CEU, Madrid, Spain, Centro de Biología Molecular "Severo Ochoa", CSIC-Universidad Autónoma de Madrid, 28049 Madrid, Spain, and BASF Bioresearch Corporation, Worcester, Massachusetts 01605

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The reaction of platinum salts with bis(naphthalimide), compound **1**, yielded two Pt−bis(naphthalimide) complexes, compounds **2** and **3** which differ from each other in their leaving groups being 1,1-cyclobutane dicarboxylate or chloride, respectively. The testing of the cytotoxic activity of compounds **2** and **3** against several tumor cell lines indicated that both compounds may be endowed with important antineoplastic properties since they circumvent cisplatin resistance. At similar rates of DNA platination ($r_b = 0.025$), compounds **2** and **3** unwind supercoiled pUC8 DNA by (48 ± 2)°. Altogether, these data suggest (i) that the cytotoxic activity of compounds **2** and **3** may be due to a combined effect of platination and intercalation and (ii) that the bis(naphthalimide) ligand is a suitable "carrier" that favors DNA targeting by *cis*-Pt(II) centers.

Introduction

Cisplatin, *cis*-diamminedichloroplatinum(II), is an anticancer drug which has been extensively studied.[1] The primary mechanism of the cytotoxicity of cisplatin and its analogues is due to covalent cross-linking of cellular DNA.[2] However, the development of cellular resistance to cisplatin in mammalian cells is a common occurrence.[1] This resistance can take three main forms: (i) increased inactivation of the drug by repair of platinum DNA lesions, (ii) increased inactivation of the drug by elevated levels of cellular low molecular weight thiols, particularly glutathione, and (iii) decreased cellular uptake of the drug. The fact that cisplatin binds to DNA has prompted efforts to increase DNA targeting by incorporation of platinum into suitable "carrier" molecules such as acridine orange,[3] anthraquinone,[4] 9-anilinoacridine,[5] and other intercalators.[6−8]

A new class of bisintercalators formed by bridging two naphthalimide moieties with an alkylamino linker has been previously described.[9] These bis(naphthalimide)s have an increased cytotoxicity over mono(naphthalimide)s. Currently bis(naphthalimide)s are being assessed in clinical trials.[10,11] In the present work, platinum was incorporated into the alkylamino linker of a representative bis(naphthalimide), compound **1** (Figure 1). The reaction of platinum salts with **1** resulted in two Pt−bis(naphthalimide) complexes, compounds **2** and **3**, having CBDCA and Cl^- as leaving groups, respectively (Figure 1). The cytotoxicity data reported here show that compounds **2** and **3** are able to circumvent cisplatin resistance. Moreover, DNA binding data suggests that the combined effect resulting from platination and intercalation might be in part responsible for the cytotoxic activity of compounds **2** and **3**.

Results and Discussion

Synthesis and Characterization of the Pt−Bis(naphthalimide) Complexes. The reaction between *cis*-Pt(CBDCA)$(DMSO)_2$ or K_2PtCl_4 and L, compound **1**, yielded compounds **2** and **3**. The microanalytical data (see Experimental Details) are consistent with the proposed empirical formulas $C_{36}H_{32}N_4O_8Pt$ and $C_{30}H_{26}N_4O_4PtCl_2$, in agreement with the structures PtLCBDCA or $PtLCl_2$, respectively.

The ^1H NMR parameters of compounds **2** and **3** are shown in the Experimental Details section. The downfield shift observed for the NH groups (2.59 ppm in **1**; 6.79 ppm in **2**; 6.49 in **3**) indicates that these groups are coordinated to the platinum atom. The same effect is observed for the methylene groups. The aromatic protons are not significantly shifted with respect to the ligand. In compound **2**, the appearance of CBDCA signals at 2.82 and 1.63 ppm indicates that this group acts as a bidentate chelate ligand through the two oxygen atoms.[12]

Cytotoxic Activity. Table 1 shows the IC_{50} values of complex **2**, complex **3**, *cis*-DDP, and bis(naphthalimide) ligand, **1**, against several tumor lines sensitive to *cis*-DDP (A2780 and CH1) and resistant to *cis*-DDP (A2780cisR, CH1cisR, and Pam 212-*ras*) and normal cells (Pam 212). It may be observed that compound **2** has IC_{50} values between 0.28 and 158 μM having a cytotoxic activity slightly lower than compound **3**, which shows IC_{50} values between 0.25 and 158 μM. Table 1 also shows that compounds **2** and **3** are approximately 1.8- and 2.7-fold, respectively, more active than *cis*-DDP in Pam 212-*ras* murine keratinocytes resistant to *cis*-DDP (IC_{50} values of 89 and 62 μM, respectively, being the IC_{50} value of *cis*-DDP is 165 μM). Moreover, compounds **2** and **3** are able to circumvent *cis*-DDP resist-

* Corresponding author.
[†] Universidad Autónoma de Madrid.
[‡] Universidad San Pablo CEU.
[§] CSIC-Universidad Autónoma de Madrid.
[||] BASF Bioresearch Corp.

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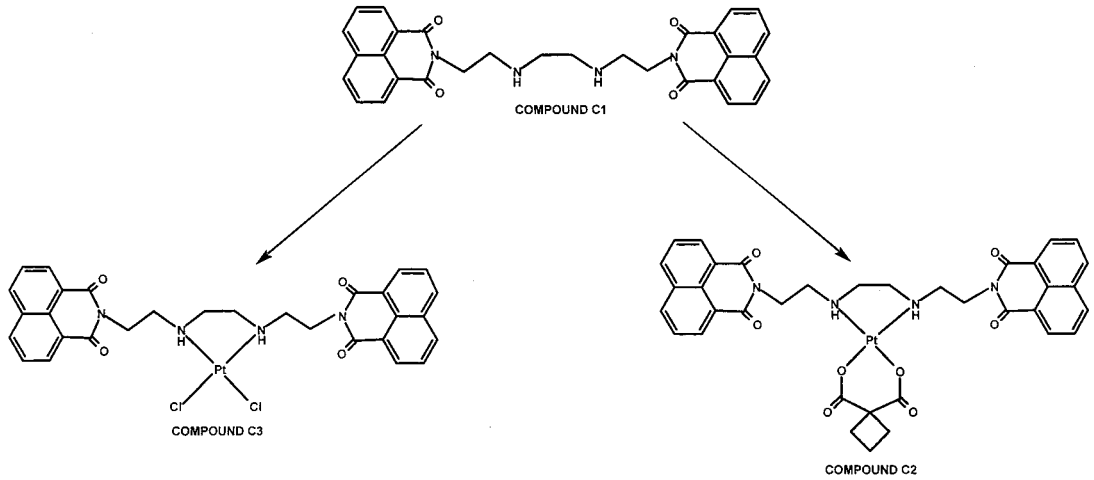

Figure 1. Structures of compounds **1**, **2**, and **3**.

Table 1. IC_{50} Mean Values Obtained for Compounds **1**, **2**, and **3** and *cis*-DDP against Several Human Ovarian Carcinoma Cell Lines and Normal and Transformed Murine Keratynocites[a]

	cell line panel ($IC_{50} \pm$ SD)					
	A2780	A2780cisR	CH1	CH1cisR	Pam 212	Pam 212-*ras*
compound **1**	>10	>100	>10	>100	140 ± 3	160 ± 6 (1.1)
compound **2**	0.28 ± 0.01	1.00 ± 0.10 (3.1)	0.35 ± 0.04	0.60 ± 0.03 (1.7)	158 ± 7	89 ± 4 (0.6)
compound **3**	0.25 ± 0.01	0.80 ± 0.04 (3.2)	0.27 ± 0.03	0.45 ± 0.02 (1.7)	5.20 ± 0.03	10.40 ± 0.6 (0.4)
cis-DDP	0.30 ± 0.02	3.30 ± 0.05 (11)	0.10 ± 0.01	0.65 ± 0.05 (6.5)	4.40 ± 0.03	39.60 ± 0.8 (1.4)

[a] SD = standard deviation. The data in parentheses are resistance factors: IC_{50} resistant line/IC_{50} parent line.

ance in human ovarian tumor cell lines A2780cisR and CH1cisR (resistance factors defined as IC_{50} resistant line/IC_{50} parental line of 3.6 and 1.7 and of 3.2 and 1.7, respectively, versus 11.0 and 6.5 for *cis*-DDP). Because CH1cisR cells are primarily resistant to *cis*-DDP through enhanced DNA repair/tolerance,[13] the better resistance factors shown by compounds **2** and **3** in relation to *cis*-DDP against these cell line suggest that the DNA adducts formed by both compounds are different from those formed by *cis*-DDP. However, it should be pointed out that in the cell lines CH1 and CH1cisR the existence of better resistance factors for compounds **2** and **3** relative to *cis*-DDP may be related to the fact that the Pt−bis(naphthalimide) compounds are less active than *cis*-DDP against *cis*-DDP-sensitive CH1 cells. Thus, their absolute cytotoxic activity could be not much higher than that of *cis*-DDP. Interestingly, the data of Table 1 show that ligand **1** displays a low level of cytotoxic activity in all the cell lines tested, suggesting that the DNA adducts formed by the bis(naphthalimide) ligand are easily repaired in CH1cisR cells.

It has been reported that Pam 212-*ras* cells are resistant to *cis*-DDP (>50%) when compared to the parental line, Pam 212.[14] Our findings are consistent with this observation and show the Pam 212-*ras* cells to be 54% less sensitive to *cis*-DDP than the parental line. The Pam 212-*ras* line has been reported to be resistant (>25%) to doxorubicin also.[14] Like doxorubicin, the bis(naphthalimide)s are DNA intercalators.[10] In our hands this line was 14% resistant to bis(naphthalimide) (see Table 1). Interestingly, compounds **2** and **3** were more active against the Pam 212-*ras* line than either *cis*-DDP or the bis(naphthalimide). Furthermore, compounds **2** and **3** showed a preferred activity against the *ras*-transformed cell line compared to the parental line (resistance factor defined as IC_{50} Pam 212-*ras* line/IC_{50} Pam 212 line of 0.6 and 0.4, respectively, versus 1.5 for *cis*-DDP). Thus, the results from the cytotoxicity tests indicate that compounds **2** and **3** may be considered as potential antitumor agents because they show a good in vitro therapeutic index and are able to circumvent resistance to *cis*-DDP in the cell lines tested.

Platinum Binding to DNA. Figure 2 shows the percentage of platinum bound to DNA at different times after addition of compound **2** or **3** or *cis*-DDP. Compound **3** has a faster rate of DNA binding than compound **2** and *cis*-DDP (55% vs 35% vs 27%, respectively, at 5 h). After 48 h of incubation, most of the *cis*-Pt(II) centers of compound **3** and *cis*-DDP are bound to DNA. In contrast, after 24 h of incubation, only 60% of the *cis*-Pt(II) centers of compound **2** are bound to DNA. These data suggest that in compound **3** intercalation of the bis(naphthalimide) ligand may favor DNA platination. However, the lower rate of DNA binding of compound **2** may be related with a slow rate of hydrolysis of the CBDCA leaving group relative to the Cl^- leaving group of compound **3**.

Interaction of Pt−Bis(naphthalimide) Complexes with Supercoiled DNA. The effect of the binding of compounds **2** and **3** on supercoiled DNA was determined by the ability of the compounds to alter the electrophoretic mobility of the ccc and oc forms of pUC8 plasmid. Figure 3 shows the electrophoretic mobility of native pUC8 DNA and pUC8 DNA incubated with compounds **1**, **2**, and **3** and *cis*-DDP at $r_b = 0.010$, 0.025, 0.050, 0.100, 0.150, 0.200, 0.250, 0.300, and 0.400 (panels A, B, C, and D; lanes: 2, 3, 4, 5, 6, 7, 8, 9, and 10). It has been reported that at increasing r_b the rate of migration of the ccc DNA band decreases until it comigrates with the oc DNA band so that the r_b at the coalescence point corresponds to the amount of platinum needed for complete removal of all supercoils from

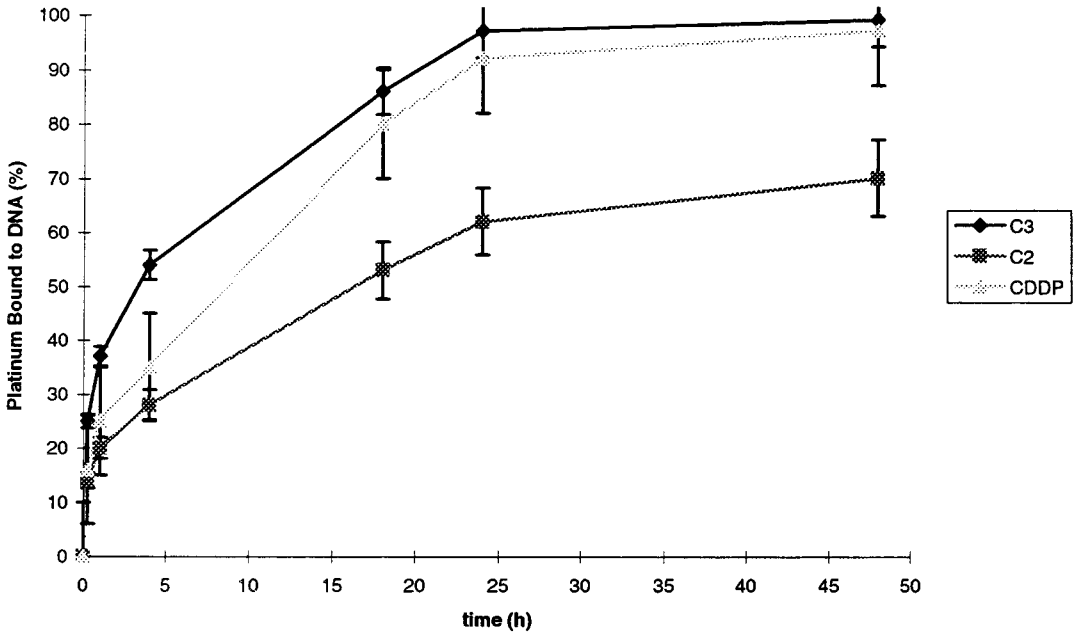

Figure 2. Kinetics of platination of pUC8 DNA by compounds **2** and **3** and *cis*-DDP.

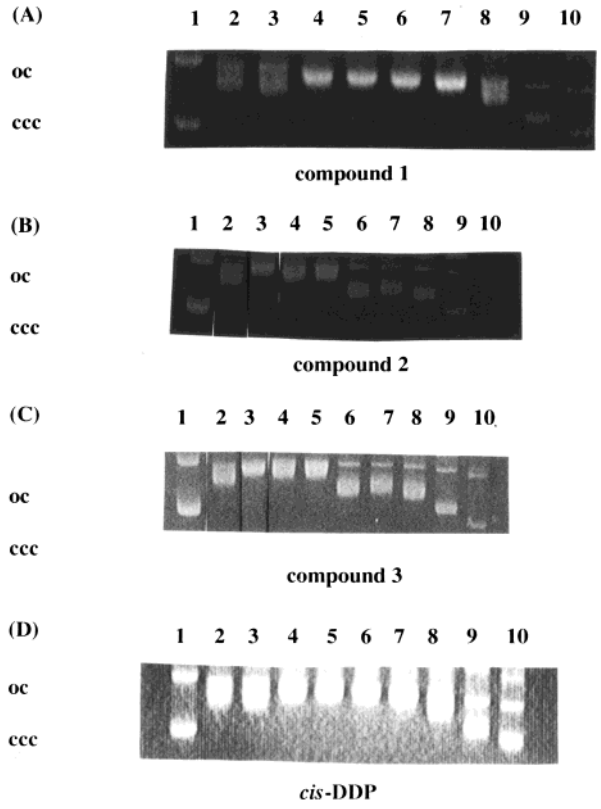

Figure 3. Changes in electrophoretic mobility of the ccc (covalently closed circular) and oc (open circular) forms of pUC8 plasmid DNA modified by compounds **1**, **2**, and **3** and *cis*-DDP (panels A, B, C, and D, respectively) at $r_b = 0.010$ (lanes 2), 0.025 (lanes 3), 0.050 (lanes 4) 0.100 (lanes 5), 0.150 (lanes 6), 0.200 (lanes 7), 0.300 (lanes 8), and 0.400 (lanes 9). Lanes 1: control unmodified pUC8 DNA.

DNA.[15] The DNA unwinding angle, ϕ, can be calculated from the following equation[16]

$$\phi = 18\sigma/r_b(c)$$

where σ is the superhelical density of the plasmid and $r_b(c)$ is the molar ratio of platinum bound per nucleotide at the coalescence point.

As can be seen in Figure 3, the $r_b(c)$ value for compounds **2** and **3** was 0.025 (panels B and C, lane 3) and gave a ϕ value of (48 ± 2)° under our experimental conditions ($\sigma = -0.067$). For compound **1** and *cis*-DDP, the calculated $r_b(c)$ values were 0.050 and 0.100 (panels A and D; lanes 4 and 5), which rendered ϕ values of (24 ± 1)° and (12 ± 0.6)°, respectively. The ϕ value of about 12° obtained for *cis*-DDP is in agreement with previously reported data.[16,17] Since the ϕ value obtained for compounds **2** and **3** is substantially higher than the ϕ values obtained for compound **1** and *cis*-DDP, it is most likely that the DNA binding mode of compounds **2** and **3** results from a combined effect of intercalation and platination.

Combination of intercalation/platination was confirmed after agarose gel electrophoresis, in the presence of chloroquine, of the complexes formed by incubation of pUC8 DNA with compounds **2** and **3** at 37 °C for 24 h (Figure 4; lanes: 1, 2, 3, and 4). The slowest moving band of each lane represents the open circular DNA (oc form). The other nine bands of each lane are all covalently closed circular DNA (ccc forms or topoisomers). Figure 4 shows that at $r_i = 0.1$, compounds **2** and **3** produce stronger alterations than the bis(naphthalimide) ligand, **1**, on the mobility of the oc form and the ccc forms (lanes 2 and 3 versus lane 4). Moreover, compound **3** produces more drastic alterations on the mobility of the oc form and the ccc forms than compound **2** (lane 2 versus lane 3), as expected from the fact that after 24 h of incubation at $r_i = 0.1$ the resulting r_b is 0.096 for compound **3** and 0.06 for compound **2** (see Figure 2). It should be noted that adjacent bands within a group of circular covalently closed pUC8 forms differ in the topological winding number, α, by 1.[18] As expected from previously reported data,[9] comparison of the results of lane 4 versus lane 1 of Figure 4 indicates that the DNA association constant of the bis(naphthalimide) ligand is much higher than that of chloroquine because complete dissociation of DNA−compound **1** complexes

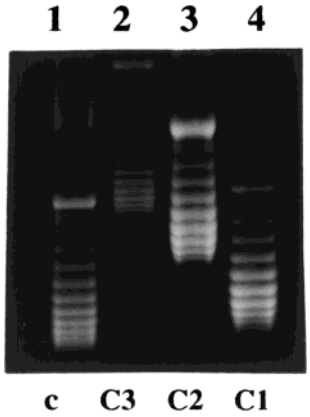

Figure 4. Resolution of DNA topoisomers of pUC8 plasmid incubated with the Pt−bis(naphthalimide) compounds at $r_i = 0.1$ after agarose gel electrophoresis with chloroquine (1.25 μg/mL). Lane 1: control unmodified pUC8. Lane 2: compound **3**:pUC8 complexes. Lane 3: compound **2**:pUC8 complexes. Lane 4: compound **1**:pUC8 complexes.

is not produced under the experimental conditions used (excess of chloroquine). Because the nine bands of DNA topoisomers of pUC8 plasmid incubated with compound **1** migrate slower than those of control pUC8 plasmid, it may be concluded that the bis(naphthalimide) ligand negatively twists supercoiled DNA.[18] Interestingly, pUC8 DNA modification by compounds **2** and **3** induces a much higher delay in the mobility of the topoisomers relative to the bis(naphthalimide) alone. Thus, most likely, compounds **2** and **3** not only act as intercalators as the bis(naphthalimide) ligand does but also as DNA platination agents. It is well-known that binding of the *cis*-Pt(II) center produces uncoiling of supercoiled DNA.[15]

Many DNA intercalators such as ethidium, phenylquinoleine, phenylbenzimidazole, and dinitrobenzene have been coupled to a *cis*-Pt(II) center. However, in most cases, the synthesized platinum complexes have shown similar cytotoxicity to the corresponding intercalating ligand. Thus, it has been hypothesized that the covalent binding ability of the *cis*-Pt(II) moiety was significantly suppressed by the intercalator.[19] Interestingly, the data reported here, from the analysis of the interaction of compounds **2** and **3** with plasmid DNA, indicate that both compounds produce DNA conformational changes that result from the combined effect of platination and intercalation. However, it should be mentioned that this is not a unique effect resulting from Pt−bis(naphthalimide) combinations because a similar effect has also been observed for a Pt−acridine orange combination having lower cytotoxic activity.[20] On the other hand, the cytotoxic activity observed in compounds **2** and **3** is probably more impressive than that indicated by the comparisons with *cis*-DDP cytotoxicity because these compounds are derivatives of *cis*-dichloro(ethylenediamine)platinum(II), which is significantly less active than *cis*-DDP.[21]

In summary, the results reported here show that the bis(naphthalimide) ligand, **1**, is a suitable "carrier" molecule that favors DNA targeting by *cis*-Pt(II) centers.

Experimental Details

The infrared spectra were recorded in Nujol mulls and KBr pellets in the 4000−200 cm^{-1} range with a Perkin-Elmer 283 spectrophotometer. The samples were ground with KBr at a concentration of 2 wt % and then pressed into pellets. For the region 600−200 cm^{-1}, the samples were prepared as Nujol mulls on CsI windows. NMR spectra were recorded on a Bruker AMX-300 in DMSO-d_6. The C, H, and N analyses were performed on a Perkin-Elmer 240B microanalyzer. All solvents were purified, prior to use, by the standard methods.[22] K_2PtCl_4 was a gift from Johnson-Matthey. *cis*-$(DMSO)_2Pt$(CBDCA) was synthesized as reported previously.[23]

Synthesis of LPt(CBDCA) (2). To a solution of *cis*-$(DMSO)_2Pt$(CBDCA) (1.48 g, 3 mmol) in water (1.62 g, 3.2 mmol), ligand was added. The solution was refluxed for 9 h, the solids were filtered-off, and the liquid was concentrated under vacuum. Acetone was added to precipitate a gray solid, which was filtered off, washed with acetone, and dried in vacuum (yield 77%). Anal. Calcd for $C_{36}H_{32}N_4O_8Pt$: C, H, N. IR (ν, cm^{-1}): 3440, 1701, 1656, 1590, 547.

Synthesis of $LPtCl_2$ (3). To a solution of K_2PtCl_4 (1.33 g, 1.32 mmol) in water (1.52 g, 1.50 mmol), ligand was added. After the mixture was stirred for 5 days, the yellow solid was filtered off and washed with water, ethanol, and ether and dried in *vacuum* (yield 95%). Anal. Calcd for $C_{30}H_{26}N_4O_4PtCl_2$: C, H, N. IR (ν, cm^{-1}): 3467, 1695, 1656, 1586, 539, 332, 326.

NMR Data. Compound 1. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.43 (m, 8H, H1, H3), 7.84 (m, 4H, H2), 4.08 (m, 4H, H4), 2.75 (m, 4H, H6), 2.59 (m, 2H, NH). The H5 signal is not observed. **Compound 2**. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.49 (m, 8H, H1, H3), 7.88 (m, 4H, H2), 4.29 (m, 4H, H4), 3.06 (m, 4H, H6), 6.79 (m, 2H, NH), 2.82 (m, 4H, H7), 1.63 (m, 2H, H8). The H5 signal is not observed. **Compound 3**. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.47 (m, 8H, H1, H3), 7.87 (m, 4H, H2), 4.29 (m, 4H, H4), 3.60 (m, 4H, H5), 2.96 (m, 4H, H6), 6.42 (m, 2H, NH).

Biological Reagents and Compounds. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma Chemical Co. FCS (foetal calf serum) was supplied by GIBCO−BRL. pUC8 DNA was supplied by Sigma Co. *cis*-DDP (*cis*-diamminedichloroplatinum(II)) and chloroquine (7-chloro-4-(4-diethylamine-1-methylbutylamine)-quinoline) were purchased from Sigma Chemical Co. Compounds **1**, **2**, and **3** and *cis*-DDP were dissolved in distilled water. Stock solutions of the compounds at concentrations between 0.5 and 1.2 mg/mL were freshly prepared before use.

Cell Lines and Culture Conditions. Cultures of ovarian carcinoma cell lines (A2780, A2780cisR, CH1, CH1cisR), normal murine keratinocytes (Pam 212 cells), and murine keratinocytes transformed with v-H-*ras* oncogene and resistant to *cis*-DDP (Pam 212-*ras* cells) have been described elsewhere.[14,24,25]

Drug Cytotoxicity. Cell survival in compound-treated cultures was evaluated by the MTT method as previously reported.[26]

Formation of Compound:DNA Complexes. Formation of compound:DNA complexes was done by addition to pUC8 (density of supercoiling, $\sigma = -0.067$) of aliquots of each compound at different concentrations in 10 mM $NaClO_4$. The amount of compound added to the DNA solution was expressed as r_i (input molar ratio of Pt/nucleotide). The mixture was incubated at 37 °C for various periods of time. The fraction of unreacted compounds was separated from the mixture by precipitation of the DNA with 2.5 volumes of ethanol and 0.3 M sodium acetate, pH 4.8.

Quantitation of Pt Binding to DNA. A 20 μg/mL solution of pUC8 DNA ($\sigma = -0.067$) in 10 mM $NaClO_4$ was incubated at 37 °C with the platinum drugs at $r_i = 0.1$. Aliquots of 250 μL were collected at various times. The amount of platinum bound per nucleotide (r_b) was determined at every reaction time by total X-ray fluorescence (TXRF) using a Seifert EXTRA-II apparatus.[27]

Interaction of Compounds 2 and 3 with Supercoiled Plasmid DNA. pUC8 DNA aliquots (50 μg/mL, $\sigma = -0.067$) were incubated with the compounds at 37 °C in 10 mM $NaClO_4$ for several periods of time until achieving r_b values of 0.010, 0.025, 0.050, 0.100, 0.150, 0.200, 0.250, 0.300, and 0.400.

Aliquots of 20 μL of compound:DNA complexes containing 1 μg of DNA were subjected to 1.5% agarose gel electrophoresis for 16 h at 25 V in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8,0) as previously reported.[28] Electrophoresis was also performed in TAE buffer containing 1.25 μg/mL of chloroquine in order to resolve DNA topoisomers.[29] The experiments were repeated 4 times.

Abbreviations: CBDCA, 1,1-cyclobutane dicarboxylate; ccc, covalently closed circular DNA form; *cis*-DDP, *cis*-diammine-dichloroplatinum(II), cisplatin; FCS, fetal calf serum; DMEM, Dulbecco modified Eagle's medium; EDTA, ethylenediaminetetracetate; r_i = input molar ratio of platinum per nucleotide; r_b = molar ratio of platinum bound per nucleotide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; Tris, tris-hydroxymethylaminomethane; oc, open circular DNA form.

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