Preparation and Characterization of Platinum(II) and (IV) Complexes of 1,3-Diaminepropane and 1,4-Diaminebutane: Circumvention of Cisplatin Resistance and DNA Interstrand Cross-Link Formation in CH1cisR Ovarian Tumor Cells

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The reaction of Pt(dimethyl sulfoxide)$_2$CBDCA (CBDCA = 1,1-cyclobutanedicarboxylate) with 1,4-diaminebutane and 1,3-diaminepropane ligands yields, under certain conditions, new [Pt(diamine)$_2$]CBDCA complexes (**1a**,**b**), where the CBDCA ligand has been removed from the coordination sphere of the platinum atom by the diamine ligand, instead of forming the expected [Pt(diamine)CBDCA] complexes (**1′a**,**b**). The structure of complexes **1a** and **1′b** was solved by X-ray diffraction. Complex **1a** crystallizes in the orthorhombic system, in the noncentrosymmetric *C222* space group, with unit cell parameters: a = 20.053(2) Å; b = 8.655(2) Å, c = 5.711(3) Å; V = 991.2(6) Å^3; δ (calcd) = 1.627 mg/m^3; and R = 0.050. The Pt atom displays an unexpected distorted tetrahedral coordination with a N−Pt−N inner bond angle equal to 81(2)° for N atoms of the same 1,3-propanediamine ligand and a N−Pt−N bond angle for different ligands equal to 135.4(9)°. Complex **1′b** crystallizes in the monoclinic system, in the centrosymmetric $P2_1/c$ space group, with unit cell parameters: a = 6.007(2) Å; b = 15.336(4) Å, c = 13.232(5) Å; β = 101.90(3)°; V = 1192.8(7) Å^3; δ (calcd) = 2.369 mg/m^3; and R = 0.067. Cytotoxicity data show that of all the synthesized compounds, only complexes **1′a** and **1′b** exhibit remarkable cytotoxic properties. Thus, in contrast with carboplatin (*cis*-diammine-1,1-cyclobutane dicarboxilatoplatinum(II)), compounds **1′a** and **1′b**, which also contain the CBDCA ligand, are able to circumvent cisplatin (*cis*-diamminedichloroplatinum(II)) resistance in several tumor cells. Moreover, after 24 h of incubation of CH1cisR ovarian tumor cells with 10 μM of compounds **1′a** and **1′b**, the level of DNA interstrand cross-links (ICLs) induced by compounds **1′a** and **1′b** is 3.3 and 3.8 times higher, respectively, than that of carboplatin and 3.5 and 4.0 times higher, respectively, than that of cisplatin. Interestingly, under the same conditions, the intracellular accumulation of compounds **1′a** and **1′b** is similar to that of carboplatin and cisplatin. However, the extent of binding to DNA of compounds **1′a** and **1′b** is similar to that of cisplatin but slightly higher than that of carboplatin. We propose that circumvention of cisplatin resistance in CH1cisR cells by compounds **1′a** and **1′b** might be related to its higher ability to form DNA ICLs relative to carboplatin and cisplatin.

Introduction

Polyamines are aliphatic cations that affect multiple functions essential for life such as cell proliferation, differentiation, and DNA replication.[1] Investigations using polyamine biosynthetic inhibitors indicate that alterations in cellular polyamine levels modulate normal and cancer cell growth.[2] In addition, palladium and platinum complexes of putrescine, spermine, and spermidine have shown cytotoxic activity against cancer cells.[3−5] Moreover, it has been recently observed that combinations of certain polyamine analogues with cytotoxic agents such as cisplatin [*cis*-diamminedichloroplatinum (II)] show synergy against breast cancer cells.[6] On the basis of these grounds, we report here the synthesis, characterization, and cellular pharmacology data of new platinum(II) and (IV) complexes of 1,3-diaminepropane (DAP) and 1,4-diaminebutane (PUT).

Carboplatin [*cis*-diammine-1,1-cyclobutane dicarboxylatoplatinum(II)] is a less toxic but equally efficacious cisplatin analogue. However, cisplatin and carboplatin have a similar spectrum of antitumor activity.[7] Thus, there is an urgent need to discover new platinum drugs capable of circumventing cisplatin/carboplatin resistance. The most general methods to synthesize amine platinum(II) carboxylate complexes of formula Pt(amine)(carboxylate) are (i) reaction of dichloroplatinum complex $PtCl_2$(amine)$_2$ with the disilver salt of a carboxylic acid,[8,9] or (ii) reaction of the dimethyl sulfoxide (DMSO) platinum complex Pt(DMSO)$_2$(carboxylate) with

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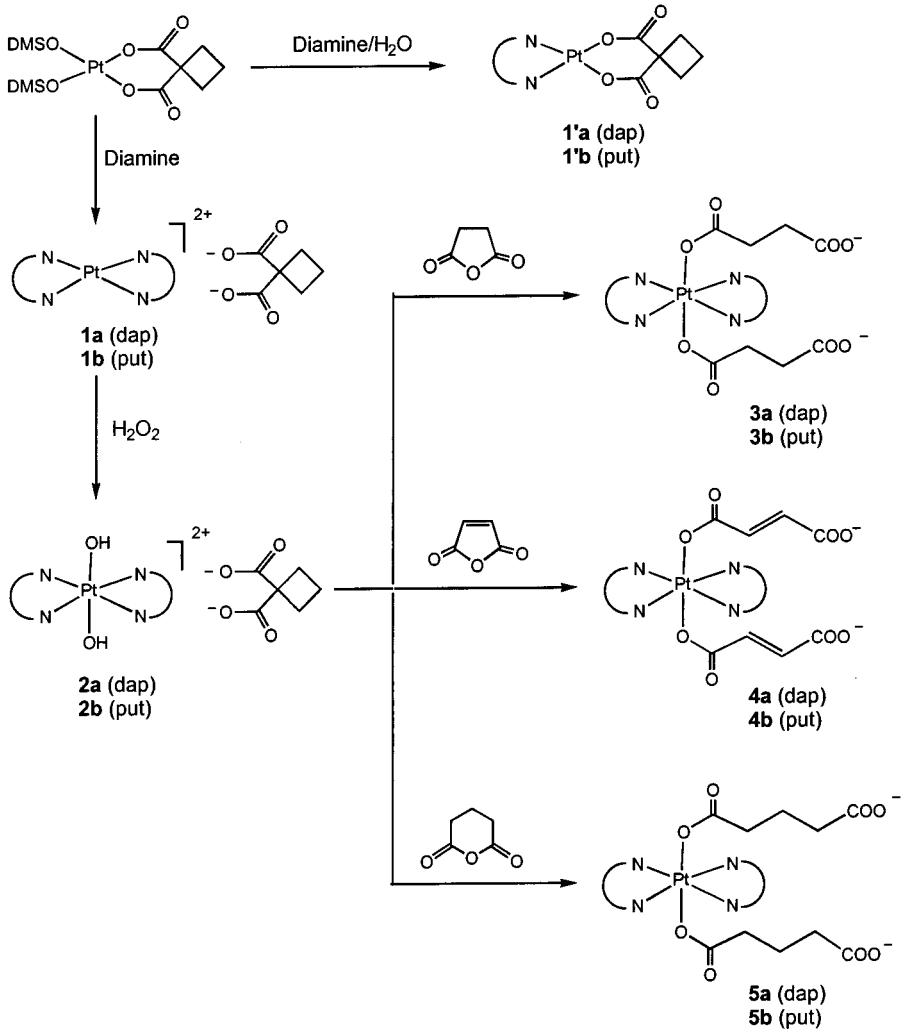
Scheme 1



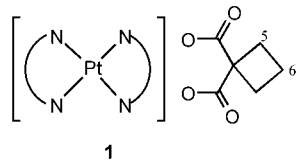
diamines.[10,11] However, our recent experiments have shown that the reaction between Pt(DMSO)$_2$CBDCA (1,1-cyclobutanedicarboxylate) and diamines such as DAP or PUT does not always afford the Pt(amine)-(CBDCA) complex. In fact, we report herein that under certain reaction conditions, it is possible to obtain new bis-amine platinum complexes of the formula [Pt-(amine)$_2$](CBDCA) where the cyclobutanedicarboxylate ligand has been removed from the coordination sphere of the platinum atom by the diamine ligand. All of these complexes have been characterized by elemental analysis, IR, and ^{1}H, ^{13}C, and ^{195}Pt nuclear magnetic resonance (NMR). An X-ray structure determination for [Pt(diamine)$_2$]CBDCA, **1a**, and Pt(diamine)CBDCA, **1′b**, confirmed the structure of the complexes with one or two diamines bonded to the platinum atom. Whereas the platinum atom has the expected square planar coordination in complex **1′b**, it shows an unexpected distorted tetrahedral coordination in complex **1a**. Recently, synthetic inorganic chemistry has taken advantage of the nucleophilic properties of hydroxo metal complexes to synthesize carboxylate metal compounds.[12] The oxidation of [Pt(diamine)$_2$]CBDCA complexes with H_2O_2 affords the [Pt(diamine)$_2$(OH)$_2$]CBDCA complexes. Reactions of these hydroxo complexes with cyclic anhydrides (succinic, maleic, and glutaric anhydrides) are also described here.

The results obtained from cytotoxicity assays of the Pt(II) and Pt(IV) complexes of DAP or PUT against pairs of cisplatin sensitive and resistant tumor cells show that only complexes **1′a** and **1′b** are endowed with remarkable cytotoxic properties. In fact, in contrast with carboplatin, compounds **1′a** and **1′b** containing the CBDCA ligand are able to circumvent cisplatin resistance in A2780cisR, CH1cisR, and Pam 212-*ras* tumor cells. DNA interstrand cross-linking assays in ovarian tumor CH1cisR cells suggest that compounds **1′a** and **1′b** might be able to overcome cisplatin resistance in this ovarian tumor cell line through interstrand cross-link (ICL) formation.

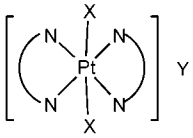
Results and Discussion

Synthesis of the Complexes. The reaction of *cis*-[Pt(DMSO)$_2$(CBDCA)] with diluted amines usually affords complexes of the formula *cis*-[Pt(amine)(CBDCA)] **1′a**,**b**, where the DMSO has been substituted by the diamine ligand.[10] However, when the amine is added directly to an aqueous solution of the *cis*-[Pt(DMSO)$_2$-(CBDCA)] complex, new ionic complexes **1a**,**b** are obtained, where two diamine ligands are coordinated to the platinum atom and the cyclobutanedicarboxylate ligand acts as anion in these complexes (Scheme 1). We have attempted several reaction conditions; however, the complexes obtained are independent of the propor-

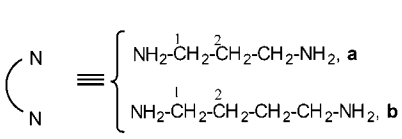
Table 1. ^{1}H NMR Data (ppm) for the Ligands and the Platinum Complexes[a]



1



2, X = OH, Y = CBDC
3, X = OOC-CH$_2$-CH$_2$-COO$^\ominus$
4, X = OOC-CH=CH-COO$^\ominus$
5, X = OOC-CH$_2$-CH$_2$-CH$_2$-COO$^\ominus$



a: NH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$
b: NH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$

	H1	H2	H5	H6	H8	H9	H10
DAP	2.73, m, 4 H	1.66, m, 2 H					
1a	2.77, m (40.41),[a] 8H	1.77, m, 4H	2.30, t(7.9), 4H	1.77, m, 2H			
1′a	2.62, m br, 4H	1.74, m br, 4H	2.81, t (7.6), 4H	1.84, q (7.6), 2H			
2a	2.71, m(39.10),[a] 8H	2.03, m, 4 H	2.31, t (7.9), 4 H	1.80, q (7.9), 2H			
3a	2.69, m, 8 H	2.05, m, 4 h			2.71, m, 4 H	2.51, m, 4 H	
4a	2.68, m, 8 H	2.05, m, 4 H			6.48, m, 2 H	5.95, m, 2 H	
5a	2.62, m, 8 H	2.05, m, 4 H			2.73, m, 4 H	2.42, m, 4 H	2.53, m, 4 H
PUT	2.61, m, 4H	1.44, m, 4H					
1b	2.85, m (47.70),[a] 8H	1.99, m, 8H	2.30, t (7.9), 4H	1.80, q (7.9), 2H			
1′b	2.68, m br, 4H	1.93, m br, 4H	2.83, t (7.7), 4H	1.87, q (7.7), 2H			
2b	2.98, m (40.50),[a] 8H	1.87, m, 8H	2.30, t (8.0), 4H	1.80, q (8.0), 2H			
3b	2.89, m, 8H	1.79, s br, 8H			2.75, m, 4H	2.55, m, 4H	
4b	2.93, m (35.82),[a] 8H	1.83, s br,8H			6.52, m 2H	6.00, m, 2H	
5b	2.86, m, 8H[b]	2.00, s br, 8H			2.81,[b] 4H	2.40, t (7.9), 4H	2.53, t (7.9), 4H

[a] J (^{1}H−^{1}H) in Hz. [b] J (^{195}Pt−^{1}H) in Hz. Overlapped signals. s br = broad singlet, m br = broad multiplet, t = triplet, q = quintuplet, and m = multiplet.

tion of diamine−platinum complex used; they are only dependent on the concentration of the diamine ligand.

The oxidation of platinum(II) complexes **1a**,**b** to obtain the platinum(IV) dihidroxo complexes **2a**,**b** with two hydroxyl groups in the axial position was carried out by the habitual method with hydrogen peroxide (33%) in the dark at 60 °C.[13]

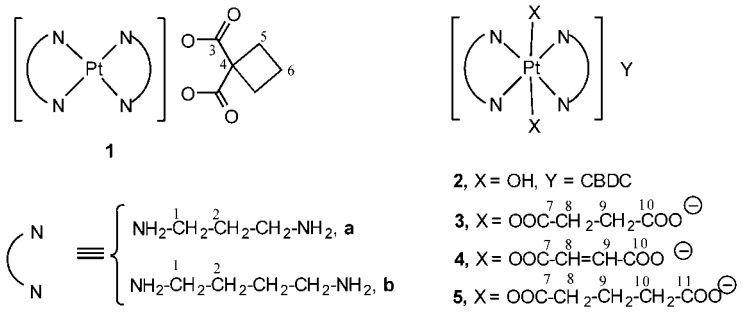
The carboxylation reaction can be used to prepare complexes containing ligands with pendant functional groups that would be difficult to introduce by substitution reactions.[14] This reaction with cyclic anhydrides is more difficult than in the case of acyclic anhydrides. We have attempted different conditions with different solvents, high temperatures, and long reaction times. When the reaction of dihydroxo complexes **2a**,**b** and different cyclic anhydrides (see Scheme 1) at reflux for 2 days in argon atmosphere was carried out, we obtained the corresponding complexes with the general formula $PtL_2(carboxylate)_2$ where the two carboxylations of the two PtOH groups have taken place.

Structural Characterization. All compounds were characterized by elemental analysis, infrared spectroscopy, NMR spectroscopy, mass spectrometry, molar conductivity, and in the case of complexes **1a** and **1′b** by X-ray diffraction. The elemental analysis for all compounds was in good agreement with the empirical formula proposed (±0.4%).

The infrared spectra of **1a**,**b** show a typical pattern expected for noncoordinated carboxylate ligands (see Experimental Section).[15] However, complexes **1′a**,**b**, where CBDCA is coordinated to the platinum atom, show a shift to lower frequencies of ν_{as}(CO) and ν_s(CO) vibrations at 1596 and 1377 cm^{-1}, respectively, for complex **1′a** and at 1597 and 1385 cm^{-1}, respectively, for complex **1′b**, typical of CBDCA when it acts as a chelate through the two oxygen atoms. The NMR spectra were assigned on the basis of chemical shift, spin−spin coupling information, heteronuclear 2D correlation spectroscopy,[16] and for quaternary carbon atoms, by using the heteronuclear Overhauser effect.[17] The ^{1}H parameter was confirmed by selective proton decoupling. The ^{1}H NMR data (Table 1) clearly indicate the presence of two diamine ligands per carboxylate for complexes **1a**,**b**. The chemical shift for H5 of 2.30 ppm in **1a**,**b** is typical for free CBDCA, whereas H5 in complexes **1′a** and **1′b** is highly deshielded (2.81 and 2.83 ppm, respectively) as a consequence of the coordination to the platinum atom.[18] The deshielding observed for the amine protons with respect to the free amine and the platinum satellites (J(^{195}Pt−^{1}H) = 40.41 Hz in **1a** and 47.70 Hz in **1b**) for H1 confirms that the amine is bonded to the platinum atom as a chelate ring. The appearance of only two broad signals for these methylene groups is in good agreement with the fast conformational equilibrium of the chelate ring.[19] The ^{13}C NMR data (Table 2) show similar chemical shifts for complexes **1a**,**b** and **1′a**,**b**. ^{195}Pt NMR data (Table 3) show one signal at −2836 and −2749 ppm for **1a** and **1b**, respectively, typical for a PtN_4 environment. In contrast, ^{195}Pt NMR spectra of complexes **1′a** and **1′b** show a signal at −1923 and −1869 ppm, respectively, which is typical for a PtN_2O_2 enviroment.[20] Molar conductivity (Λ_M = 126 and 115 cm^2 ohm^{-1} mol^{-1} for **1a** and **1b**, respectively) indicates the presence of two ions in the solution.[21] Compounds **1′a** and **1′b** do not show any conductivity in the same conditions.

The structure of complexes **1a** and **1′b** was confirmed by X-ray diffraction. The crystal structure of compounds **1a** and **1′b** is shown in Figures 1 and 2, respectively, together with the atomic numbering scheme and se-

Table 2. ^{13}C NMR Data (ppm) for the Ligands and the Platinum Complexes



	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
DAP											
1a	41.83	26.61	181.99	57.36	29.73	14.72					
1′a	41.75	26.88	181.46	55.54	30.66	14.42					
2a	38.69	25.23	181.95	57.04	29.54	14.72					
3a	39.23	24.74					181.46	30.35	30.74		179.03
4a	39.27	24.72					180.21	136.24	121.20		178.88
5a	39.27	24.71					178.61	41.82	20.20	33.65	178.99
PUT	41.83	31.05									
1b	45.27	25.03	181.97	57.36	29.70	14.71					
1′b	45.91	24.95	181.12	55.63	30.40	14.63					
2b	44.48	24.57	182.01	57.14	29.57	14.70					
3b	45.50	24.12					181.23	31.21	32.05		178.85
4b	46.14	24.12					173.58	123.36	135.66		172.44
5b	45.41	24.80					180.12	40.33	21.07	34.63	177.24

Table 3. ^{195}Pt NMR Data (ppm) of the Platinum Complexes

1a	−2836	**3a**	752	**1b**	−2749	**3b**	810
2a	440	**4a**	769	**1′b**	−1869	**4b**	843
1′a	−1923	**5a**	748	**2b**	502	**5b**	825

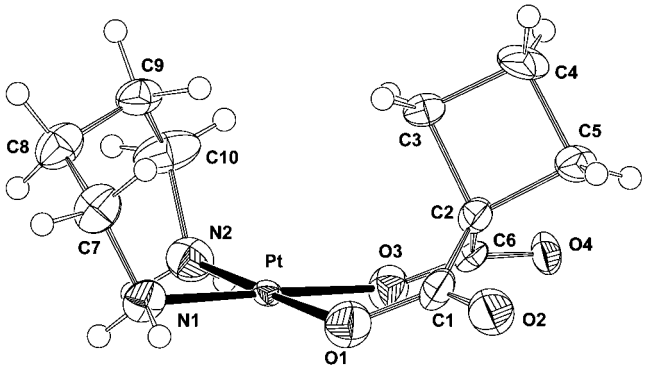


Figure 1. Molecular structure of compound $[Pt(PUT)_2]$-CBDCA **1a**. ORTEP, 50% ellipsoids. Disorder and H atoms were omitted for clarity. Pt−N1 = 2.09(2) Å; Pt−N2 = 2.02(1) Å; O1−C11 = 1.27(3) Å; O2−C11 = 1.25(3) Å; N1−Pt−N2 = 81(2)°.

lected bond distances and angles. The X-ray parameters of compounds of **1a** and **1′b** are summarized in Table 4.

The structure of complex **1a** consists of two separate ions. The cation is formed by the platinum atom bonded to four nitrogen atoms, two from each diamine ligand, in a six-member chelate ring conformation (Figure 1).

Selected bond lengths and angles for compound **1a** are shown in Table 5. The Pt atom shows an square-planar geometry distorted toward tetrahedral with the N−Pt−N bond angle equal to 81(2) and 135.4(9)° for N atoms of the same and different ligand, respectively. The Pt−N bond lengths of 2.09(2) and 2.02 (1) Å for Pt−N1 and Pt−N2, respectively, are in the normal range of those found in other similar complexes.[22] The 1,1′-

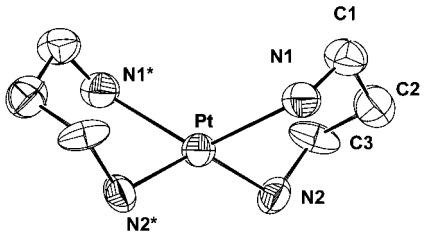


Figure 2. Molecular structure of compound [Pt(DAP)CBDCA] **1′b**. H atoms were omitted for clarity. Pt−O1 = 2.008(6) Å; Pt−O3 = 1.986(7) Å; Pt−N1 = 1.989(8) Å; Pt−N2 = 1.994(7) Å; O1−C1 = 1.30(1) Å; C1−O2 = 1.250(8) Å; O3−C6 = 1.289(9) Å; O4−C6 = 1.197(9) Å; O1−Pt−O3 = 91.9(3)°; O1−Pt−N1 = 88.7(3)°; N1−Pt−N2 = 92.4(3)°; N2−Pt−O3 = 87.1(3)°.

cyclobutanedicarboxylate anion is hydrogen-bonded to two different $[Pt(DAP)_2]^{2+}$ ions. The N(2)···O(1) length is equal to 3.01(2) Å.

The structure of **1′b** consists of discrete neutral molecules separated by van der Waals distances. The platinum atom is bonded to nitrogen atoms from a diamine ligand and two oxygen atoms from the CBDCA ligand, both in a chelate ring conformation. The platinum center shows a square-planar structure (Figure 2).

Selected bond lengths and angles for compound **1′b** are shown in Table 6. Coordination of CBDCA to the platinum atom does not modify the bond distances significantly. Pt−N bond distances of 1.989(8) and 1.994(7) Å for Pt−N1 and Pt−N2, respectively, are slightly shorter than in compound **1a**, where the trans position is occupied by other diamine but is in the normal range for these complexes.[21]

After oxidation, the most characteristic signal that identifies these complexes in the IR spectrum is the Pt−O stretching vibration[13] at 557 and 573 cm^{-1} for **2a** and **2b**, respectively. ^1H and ^{13}C NMR spectra show a slight shift of the signals corresponding to the amine ligands with respect to complexes **1a**,**b**, whereas the carboxylate signals show almost the same chemical

Table 4. Crystal Analysis Parameters of Complexes **1a** and **1′b**

	compd 1a	compd 1′b
	Crystal Data	
formula	$C_{12}H_{26}N_4O_4Pt$	$C_{10}H_{18}N_2O_4Pt$
cryst size (mm)	0.1 × 0.1 × 0.2	0.2 × 0.3 × 0.3
habit	prismatic	prismatic
symmetry	orthorhombic, *C222*	monoclinic, $P2_1/c$
unit cell dimens		
a (Å)	20.053(2)	6.007(2)
B (Å)	8.655(2)	15.336(4)
c (Å)	5.711(3)	13.232(5)
β (deg)		101.90(3)
packing		
V (Å^3), *Z*, *Dc* (g cm^{-3})	991.2(6), 2, 1.627	1192.8(7), 4, 2.369
M, *F*(000), μ (cm^{-1})	485.46, 472, 7.095	425.35, 808, 11.769
	Experimental Data	
no. of rflns		
measd	1579	3478
indep	1374	3478
obsd	1374	3428
range of *hkl*	0 + 28, 0 + 12, 0 + 8	−8 to +8, 0 + 21, 0 + 18
value of R_{int}	0.051	0.000
	Solution and Refinement	
largest diff peak and hole (eÅ^{-3})	0.628 and −0.604	8.708 and −6.961
extinction coeff	0.0000(5)	0.0002(6)
absolute struct param	0.25(7)	
R, R_w	0.050, 0.115	0.067, 0.165

Table 5. Selected Bond Legths and Angles for **1a**

distances (Å)		angles (deg)	
Pt−N1	2.09(2)	N1−Pt−N2	81(2)
Pt−N2	2.02(1)		
O1−C11	1.27(3)		
O2−C11	1.25(3)		

Table 6. Selected Bond Legths and Angles for **1′b**

distances (Å)		angles (deg)	
Pt−O1	2.008(6)	O1−Pt−O3	91.9(3)
Pt−O3	1.986(7)	O1−Pt−N1	88.7(3)
Pt−N1	1.989(8)	N1−Pt−N2	92.4(3)
Pt−N2	1.994(7)	N2−Pt−O3	87.1(3)
O1−C1	1.30(1)		
C1−O2	1.250(8)		
O3−C6	1.289(9)		
O4−C6	1.197(9)		

shift, which indicates that the CBDCA ligand is still not coordinated to the platinum atom. ^{195}Pt NMR confirms that the oxidation has been produced. Both complexes show one signal at 440 and 502 ppm for **2a** and **2b**, respectively, typical of Pt(IV) with a PtN_4O_2 environment.[20] Molar conductivity (Λ_M = 105 and 142 cm^2 ohm^{-1} mol^{-1} for **2a** and **2b**, respectively) confirms again the presence of two ions in the solution.[23]

The carboxylate complexes show four strong bands in the IR spectra, two assignable to two different ν_{as}(CO) and the other two corresponding to the 3(CO) stretching, indicating that two different carboxylate groups are present in the complexes.[24] ^{1}H NMR spectra show the disappearance of the signals corresponding to the CBDCA group. ^{13}C NMR spectra show two different signals for the two different carboxylate groups (see Table 3), indicating that there are two COO^- terminal groups that maintain the electrical neutrality of the Pt atom. ^{195}Pt NMR confirms the PtN_4O_2 environment for these complexes.

Cytotoxic Activity. The synthesized compounds were tested against cisplatin sensitive (A2780, CH1) and cisplatin resistant (A2780cisR, CH1cisR) human ovarian tumor cells[25,26] and normal (Pam 212) and transformed (Pam 212-*ras*) murine keratynocites.[27] Table 7 shows the IC_{50} values obtained for the Pt(II) and Pt(IV) complexes of DAP and PUT and carboplatin and cisplatin against the above-mentioned tumor cell lines. All of the compounds with the exception of **1′a** and **1′b** showed IC_{50} values higher than 200 μM (the highest drug concentration tested). It may be observed that compound **1′a** exhibits IC_{50} values between 0.30 and 85 μM having a cytotoxic activity similar to that of compound **1′b** that shows IC_{50} values between 0.32 and 100 μM. Interestingly, compounds **1′a** and **1′b** are able to circumvent cisplatin resistance in human ovarian tumor cell lines A2780cisR and CH1cisR (resistance factor, RF, defined as IC_{50} resistant line/IC_{50} parental line of 3.3 and 1.8 and of 2.3 and 1.8, respectively, vs 18.0 and 4.2 for cisplatin). However, with carboplatin, there was not evidence of circumvention of cisplatin resistance in the pairs of cell lines A2780/A2780cisR (RF = 10) and CH1/CH1cisR (RF = 3.6); moreover, it exhibited IC_{50} values significantly higher than cisplatin against these cell lines. Because CH1cisR tumor cells show acquired resistance to cisplatin as a result of enhanced DNA repair,[26] these data suggest that compounds **1′a** and **1′b** form some type of DNA adduct that is different from the DNA adducts that are preferentially formed by cisplatin or carboplatin.[28]

Table 7 also shows that compounds **1′a** and **1′b** show significant cytotoxic activity against Pam 212-*ras* cells overexpressing H-*ras* oncogene[29] (IC_{50} values of 64 and 42 μM, respectively; the IC_{50} value of cisplatin is 72 μM). However, carboplatin showed poor cytotoxic activity against Pam 212-*ras* cells (IC_{50} value > 200 μM). Moreover, compounds **1′a** and **1′b** exhibit a better in vitro therapeutic index (T. I.) than cisplatin in the pair of cell lines Pam 212/Pam212-*ras* since they have a much lower toxicity in normal Pam 212 cells than in Pam 212-*ras* cells (T. I. of 1.3 and 2.4, respectively, vs 0.6 for cisplatin). Thus, the results from the cytotoxicity test suggest that compounds **1′a** and **1′b** merit further evaluation as potential antitumor agents because, in contrast with carboplatin, they show a good in vitro T. I. and are able to overcome cisplatin resistance in several tumor cell lines.

DNA ICL Formation in CH1cisR Cells. It has been reported that resistance of tumor cells to cisplatin may be associated with increased repair efficiency of DNA ICLs.[30] On the other hand, the CH1cisR line may be considered a tumor cell model in which cisplatin resistance may be studied at the level of drug−DNA adducts.[26] Thus, we have analyzed in CH1cisR cells the efficiency of ICLs formation of compounds **1′a** and **1′b** in relation to cisplatin and carboplatin.

The CH1cisR cells were exposed to a concentration of 10 μM of compounds **1′a** and **1′b** and cisplatin and carboplatin, and the cross-link indexes (see Experimental Section) induced by these platinum complexes were determined after several periods of incubation. Figure 3 shows that compounds **1′a**, **1′b**, cisplatin, and carboplatin produced increasing levels of ICLs when the period of drug treatment increased. In addition, compounds **1′a** and **1′b** induced a higher amount of ICLs than cisplatin and carboplatin at all of the periods of

Table 7. IC_{50} Mean Values (μM) Obtained for the Synthesized Platinum(II) and (IV) Complexes of DAP and PUT, Carboplatin and Cisplatin against Several Human Ovarian Carcinoma Cell Lines (A2780, A2780cisR, CH1, and CH1cisR) and Normal (Pam 212) and Transformed Murine Keratynocites (Pam 212-*ras*)

	cell line panel [IC_{50} (mM) ± SD]					
	A2780	A2780cisR	CH1	CH1cisR	Pam 212	Pam 212-*ras*
1a	>200	>200	>200	>200	>200	>200
1′a	0.30 ± 0.02	1.00 ± 0.30 (3.3)	4.5 ± 0.4	8.0 ± 0.9 (1.8)	85 ± 3	64 ± 3 (1.3)*
1b	>200	>200	>200	>200	>200	>200
1′b	0.32 ± 0.02	0.74 ± 0.05 (2.3)	3.3 ± 0.4	6.0 ± 0.7 (1.8)	100 ± 6	42 ± 2 (2.4)*
2a	>200	>200	>200	>200	>200	>200
2b	>200	>200	>200	>200	>200	>200
3a	>200	>200	>200	>200	>200	>200
3b	>200	>200	>200	>200	>200	>200
4a	>200	>200	>200	>200	>200	>200
4b	>200	>200	>200	>200	>200	>200
5a	>200	>200	>200	>200	>200	>200
5b	>200	>200	>200	>200	>200	>200
carboplatin	18 ± 2	178 ± 10 (10)	52 ± 4	185 ± 11 (3.6)	>200	>200
cisplatin	2.0 ± 0.4	36 ± 2 (18)	6.0 ± 1	25 ± 2 (4.2)	44 ± 3	72 ± 4 (0.6)*

* SD = standard deviation; () = resistance factors: IC_{50} resistant line/IC_{50} parent line; and ′ = therapeutic index: IC_{50} normal line/IC_{50} transformed line.

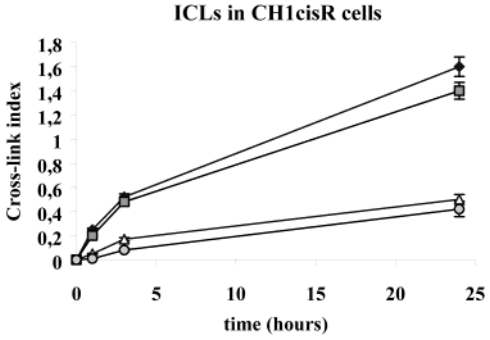


Figure 3. Alkaline elution plots showing the time course for formation of DNA ICLs in CH1cisR cells by 10 μM of compounds **1′a** (■), **1′b** (◆), cisplatin (△), and carboplatin (○). The results are expressed as means ± SD (n = 3).

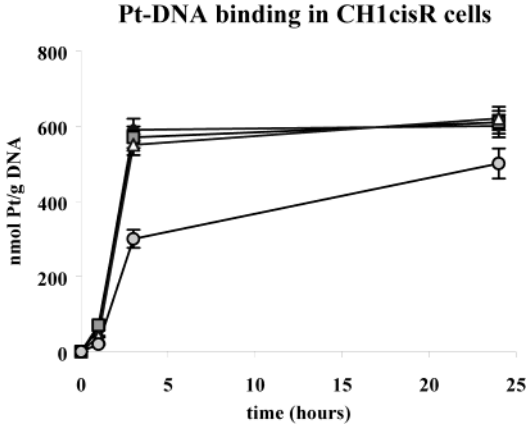


Figure 4. DNA binding kinetics of 10 μM of compounds **1′a** (■), **1′b** (◆), cisplatin (△), and carboplatin (○) in CH1cisR cells. The results are expressed as means ± SD (n = 3).

incubation tested. After 3 h of drug treatment, the cross-link index of cisplatin was 2 times higher than that of carboplatin. In addition, the cross-link index of compounds **1′a** and **1′b** was 2.8 and 3.0 times higher, respectively, than that of cisplatin and 6.0 and 6.5 times higher, respectively, than that of carboplatin. However, after 24 h of drug treatment, the cross-links index of cisplatin was similar to that of carboplatin. However, the cross-link index of compounds **1′a** and **1′b** was 3.5 and 4.0 times higher, respectively, than that of cisplatin and 3.3 times and 3.8 times higher, respectively, than that of carboplatin.

Platinum−DNA Binding in CH1cisR Cells. We wanted to know whether, relative to the total number of DNA adducts, the fraction that is ICLs is greater for compounds **1′a** and **1′b** than for cisplatin and carboplatin. So, platinum−DNA binding levels in CH1cisR cells incubated with 10 μM of compounds **1′a**, **1′b**, cisplatin, and carboplatin for 1, 3, and 24 h were determined. Figure 4 shows that the binding of compounds **1′a**, **1′b**, cisplatin, and carboplatin began to be quantifiable only after 1 h of incubation.

Platinum binding to DNA for compounds **1′a**, **1′b**, cisplatin, and carboplatin progressively increased to reach, respectively, 570, 590, 550, and 300 nmol Pt/g DNA after 3 h of incubation and 610, 600, 620, and 500 nmol Pt/g DNA after 24 h of incubation. Figure 4 also shows that in CH1cisR cells the kinetics of binding to DNA of compounds **1′a** and **1′b** is similar to that of cisplatin. In contrast, the kinetics of binding to DNA of carboplatin is slower than that of compounds **1′a**, **1′b**, and cisplatin. Altogether, the DNA binding and ICLs data of complexes **1′a** and **1′b** in CH1cisR cells indicate that both compounds have a faster kinetics of DNA binding than carboplatin, and moreover, they are able to form a higher amount of DNA ICLs than carboplatin and cisplatin.

Platinum Accumulation in CH1cisR Cells. We also looked at the intracellular platinum levels after exposure of CH1cisR cells to 10 μM of compounds **1′a**, **1′b**, cisplatin, and carboplatin for 1, 3, and 24 h. Figure 5 shows that cellular accumulation of compounds **1′a**, **1′b**, cisplatin, and carboplatin increased as a function of time in CH1cisR cells.

The intracellular levels of compounds **1′a**, **1′b**, cisplatin, and carboplatin were, respectively, 0.10, 0.16, 0.15, and 0,15 μmol/2 × 10^6 cells after 1 h of incubation and progressively increased to reach, respectively, 0.42, 0.45, 0.40, and 0,42 μmol/2 × 10^6 cells after 3 h of incubation and 0.45, 0.48, 0.47, and 0.52 μmol/2 × 10^6 cells after 24 h of incubation. These data indicate that in CH1cisR cells there are not significant differences in the kinetics of platinum accumulation of compounds **1′a**, **1′b**, cisplatin, and carboplatin.

Conclusions. In this study, we report some interesting structure−activity relationships of new platinum-

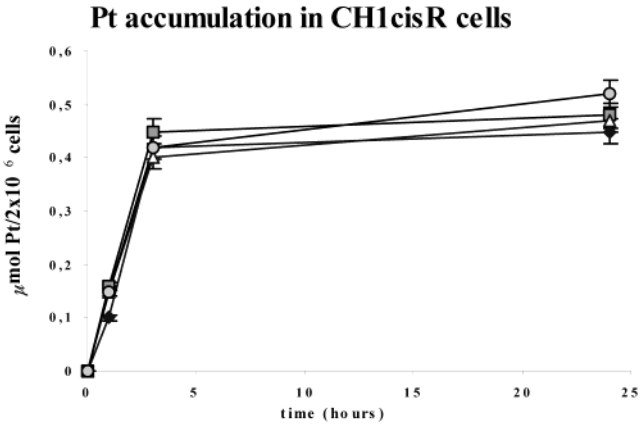


Figure 5. Platinum accumulation of 10 μM of compounds **1′a** (■), **1′b** (◆), cisplatin (△), and carboplatin (○) in CH1cisR cells. The results are expressed as means ± SD (n = 3).

(II) and (IV) complexes of DAP and PUT. The results indicate that both charged and neutral Pt(II) and Pt(IV) compounds containing two diamine ligands of DAP or PUT (compounds **1a**−**5a** and **1b**−**5b**) are devoid of cytotoxic activity. These data may be interpreted taking into account that in those complexes the formation of active species may be prevented by the two DAP or PUT diamine ligands that act as inert chelate groups. In contrast, compounds **1′a** and **1′b**, which contain CBDCA as leaving group, show remarkable cytotoxic properties and are able to circumvent cisplatin resistance. We think that compounds **1′a** and **1′b** are interesting platinum complexes considering the fact that they circumvent cisplatin resistance in a tumor cell model (CH1cisR) where platinum resistance is known to include a nonglutathione-based mechanism (increased tolerance/repair of cisplatin−DNA adducts).[31] We propose that the faster kinetics of DNA binding of compounds **1′a** and **1′b** in comparison to carboplatin and their higher efficiency of DNA ICLs formation relative to both carboplatin and cisplatin may be at least in part responsible for their biological activity in ovarian tumor CH1cisR cells. On the other hand, our DNA binding data support previously reported evidences indicating that carboplatin and cisplatin differ only in the kinetics of their interaction with DNA.[32] The structural data reported here show that compounds **1′a** and **1′b** are sterically hindered platinum complexes because the diamine ligand (DAP or PUT) is coordinated to platinum as a chelate ring (see, for instance, the X-ray structure of compound **1′b** in Figure 2). Thus, in these complexes, the ability of the *cis*-Pt(II) center to form specific types of adducts such as DNA ICLs might be increased relative to cisplatin due to the steric hindrance imposed by the diamine chelate ring. In fact, it has been previously reported that the antitumor drug oxaliplatin is able to circumvent cisplatin resistance through the formation of DNA adducts different from those formed by cisplatin.[33] Interestingly, oxaliplatin features a dicarboxylate leaving group and a bulky 1,2-diaminocyclohexane ligand. On the other hand, the faster kinetics of DNA binding shown by compounds **1′a** and **1′b** relative to carboplatin suggest that the diamine ligand ring may favor the release of the CBDCA leaving group from the *cis*-Pt(II) center. On the other hand, no significant differences have been observed in the levels of DNA binding nor ICLs indexes of compounds **1′a** and **1′b.** These results suggest that the steric hindrance imposed to the binding of the *cis*-Pt(II) center to DNA by a diamine six member chelate ring (compound **1′a**) or a diamine seven member chelate ring (compound **1′b**) is quite similar.

Experimental Section

Chemicals. DAP and PUT were purchased from Aldrich whereas K_2PtCl_4 was a gift from Johnson-Matthey Chem Ltd. Cisplatin and carboplatin were purchased from Sigma. The complexes *cis*-[Pt(DMSO)$_2$Cl$_2$] and *cis*-[Pt(DMSO)$_2$(CBDCA)] were prepared according to the reported method.[10]

Synthesis of *cis*-[Pt(amine)$_2$](CBDCA) (1). To a solution of *cis*-[Pt(DMSO)$_2$(CBDCA)] (1 mmol) in water (10 mL), amine (2.1 mmol) was added. The solution was stirred at 100 °C for 6 h, filtered off with Celite, and concentrated under vacuum. The white solid was precipitated with acetone, filtered off, washed with acetone, and dried under vacuum. Complex **1a**: Yield, 87%. IR (ν cm^{-1}): 3212, 3102, 1653, 1404, 526. Anal. ($PtC_{12}H_{26}N_4O_4$) C, H, N. Mass (m/z): 342. Λ_M = 126 cm^2 ohm^{-1} mol^{-1}. Complex **1b**: Yield, 63%. IR (ν cm^{-1}): 3162, 3072, 1626, 1564, 1431, 1410, 467. Anal. ($PtC_{14}H_{30}N_4O_4$) C, H, N. Mass (m/z): 370, 281. Λ_M = 115 cm^2 ohm^{-1} mol^{-1}.

Synthesis of Pt(amine)(CBDCA) (1′). To a solution of *cis*-[Pt(CBDCA)(DMSO)$_2$] (1 mmol) in water (10 mL), amine (1.1 mmol) in water (5 mL) was added. The solution was stirred at 100°C for 6 h, filtered off with Celite, and concentrated under vacuum. The white solid was precipitated with acetone, filtered off, washed with acetone, and dried under vacuum. Complex **1′a**: Yield, 52%. IR (ν cm^{-1}): 3225, 3104, 1596, 1377, 585, 445. Anal. ($PtC_9H_{16}N_2O_4$) C, H, N. Mass (m/z): 411. Complex **1′b**: Yield, 37%. IR (ν cm^{-1}): 3212, 3123, 1597, 1385, 588, 443. Anal. ($PtC_{10}H_{18}N_2O_4$) C, H, N. Mass (m/z): 425, 281.

Synthesis of *cis,cis,trans*-[Pt(amine)$_2$(OH)$_2$](CBDCA) (2). To a solution of **1** (1 mmol) in water (10 mL), 2 mL of 33% hydrogen peroxide (H_2O_2) was added dropwise. The solution was stirred at 60°C for 4 h in the darkness. The reaction mixture was left stirring overnight at room temperature and protected from light. The clear solution was concentrated under vacuum. The solid was precipitated with acetone, filtered off, washed with acetone, and dried under vacuum. Complex **2a**: Yield, 67%. IR (ν cm^{-1}): 3155, 3115, 1630, 1402, 557, 530. Anal. ($PtC_{12}H_{28}N_4O_6$) C, H, N. Mass (m/z): 375, 358, 342. Λ_M = 105 cm^2 ohm^{-1} mol^{-1}. Complex **2b**: Yield, 84%. IR (ν cm^{-1}): 3367, 3139, 1619, 1538, 1442, 1404, 573. Anal. ($PtC_{14}H_{32}N_4O_6$) C, H, N. Mass (m/z): 404, 387, 369, 281. Λ_M = 142 cm^2 ohm^{-1} mol^{-1}.

Synthesis of *cis,cis,trans*-[Pt(amine)$_2$(O-Succinate)$_2$] (3). To a suspension of succinic anhydride (50 mmol) in CH_2Cl_2 at 40 °C was added **2** (1 mmol) in an argon atmosphere. The solution was stirred at reflux for 24 h. The white product obtained was filtered off, washed with CH_2Cl_2, and dried under vacuum. Complex **3a**: Yield, 61%. IR (ν cm^{-1}): 3208, 3025, 1645, 1597, 1418, 1382, 479. Anal. ($PtC_{14}H_{28}N_4O_8$) C, H, N. Mass (m/z): 576, 460, 341. Complex **3b**: Yield, 57%. IR (ν cm^{-1}): 3428, 3174, 1692, 1654, 1546, 1408, 561, 453. Anal. ($PtC_{16}H_{32}N_4O_8$) C, H, N. Mass (m/z): 604, 487, 369, 281.

Synthesis of *cis,cis,trans*-[Pt(amine)$_2$(O-Maleate)$_2$] (4). To a suspension of maleic anhydride (50 mmol) in CH_2Cl_2 at 40 °C was added **2** (1 mmol) in an argon atmosphere. The solution was stirred at 30°C for 2 days. The white product obtained was filtered off, washed with CH_2Cl_2, and dried under vacuum. Complex **4a**: Yield, 51%. IR (ν cm^{-1}): 3218, 2916, 1621, 1585, 1385, 1361. Anal. ($PtC_{14}H_{24}N_4O_8$) C, H, N. Mass (m/z): 570, 456, 342. Complex **4b**: Yield, 40%. IR (ν cm^{-1}): 3055, 2919, 1635, 1588, 1431, 1373, 608. Anal. ($PtC_{16}H_{28}N_4O_8$) C, H, N. Mass (m/z): 598, 484, 370, 281.

Synthesis of *cis,cis,trans*-[Pt(amine)$_2$(O-Glutarate)2] (5). To a suspension of glutaric anhydride (50 mmol) in CH_2Cl_2 at 40 °C was added **2** (1 mmol) in an argon atmosphere. The solution was stirred at 30 °C for 2 days. The white product

obtained was filtered off, washed with CH_2Cl_2 and ether, and dried under vacuum. Complex **5a**: Yield, 42%. IR (ν cm^{-1}): 3234, 3042, 1637, 1556, 1410, 1377, 478. Anal. ($PtC_{16}H_{32}N_4O_8$) C, H, N. Mass (*m*/*z*): 602, 472, 342. Complex **5b**: Yield, 61%. IR (ν cm^{-1}): 3050, 2944, 1633, 1571, 1398, 1387, 585, 477. Anal. ($PtC_{18}H_{36}N_4O_8$) C, H, N. Mass (*m*/*z*): 630, 500, 369, 281.

IR and NMR Spectroscopies. The infrared spectra were recorded as Nujol mulls and KBr pellets in the 4000−200 cm^{-1} range using a Perkin-Elmer model 283 spectrophotometer. NMR spectra were recorded on Bruker WP-200-SY (200 MHz) and AMX-300 (300 MHz) spectrometers in D_2O as the solvent, and the chemical shifts were determined when referenced to the residual deuterated solvent peak. ^{195}Pt chemical shifts were measured relative to an external standard of Na_2PtCl_6 in D_2O.

Elemental Analyses and Mass Spectra. The C, H, and N analyses were carried out on a Perkin-Elmer 240B microanalyzer. Fast atom bombardment mass spectra (*m*/*z*) were recorded with a V. G. Autospect high-resolution spectrometer by L-SIMS techniques using *m*-NBA. Conductivity measurements were carried out on a Metrohm AGCH-9100 at 25 °C in H_2O.

Crystal Structure Determination and Refinement of Complexes 1a and 1′b. Prismatic crystals were selected and mounted on a Enraf-Nonius CAD4 four-circle diffractometer. Unit cell parameters were determined from automatic centering of 25 reflections ($12 < \theta < 21°$) and refined by the least-squares method. Intensities were collected with graphite monochromatized Mo Kα radiation, using $\omega/2\theta$ scan technique. The crystal data and details of the data collection and structure analyses are summarized in Table 4. During the data collection, three standard reflections were measured every 2 h as orientation and intensity control; significant intensity decay was not observed. Lorentz polarization was made. An absorption correction was made for **1a**, with transmission factor max 0.03 and min 0.01. The structure of **1a** was solved by direct methods, whereas the structure of **1′b** was solved by Patterson synthesis, using the SHELXS computer program[34] and refined by full-matrix least-squares method with the SHELX93 computer program.[35] The function minimized was $\sum w||Fo|^2 - |Fc|^2|^2$, where $w = [\sigma^2(I) + (0.0622P)^2]^{-1}$, and $P = (|Fo|^2 + 2|Fc|^2)/3$, *f*, *f*′, and *f*″ were taken from International Tables of X-ray Crystallography.[36] The crystal selected for compound **1a** was a twin crystal; so, all atoms with the exception of the Pt atom were located in desorder sites, and an occupancy factor of 0.5 was assumed for all nonplatinum atoms. All nonhydrogen atoms were refined anisotropically. All H atoms were computed and refined with an overall isotropic temperature factor using a riding model.

Biological Reagents. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma. The platinum compounds were dissolved in 10 mM of $NaClO_4$ in distilled water. Stock solutions of the compounds at concentrations of 1 mg/mL were freshly prepared before use.

Cell Lines and Culture Conditions. Cultures of ovarian cancer cell lines (A2780, A2780cisR, CH1, and CH1cisR), normal murine keratinocytes (Pam 212 cells), and murine keratinocytes transformed with the H-*ras* oncogene and resistant to cisplatin (Pam 212-*ras* cells) have been described elsewhere.[25−27]

Drugs Cytotoxicity. Cell survival in compound-treated cultures was evaluated by the MTT method as previously reported.[37] Platinum compounds were added to 96 microwells plates containing the cell cultures at final concentrations between 0 and 200 μM. Twenty-four hours later, cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker microplate reader 2001. IC_{50} values (compound concentration that produces 50% of cell killing) were calculated from curves constructed by plotting cell survival (%) vs compound concentration (μM). Experiments were carried out in quadruplicate.

In Vivo DNA ICL Formation. DNA ICLs in culture cells were determined by alkaline filter elution as described previously.[38] The DNA of CH1cisR cells was labeled by seeding 10^6 cells in P100 tissue culture plates and growing for 24 h in the presence of 0.03 μCi/ml [^{14}C]thymidine (specific activity, 51 mCi/mmol; Amersham International). A plate of cells to be used as an internal standard in the assay was labeled overnight with 0.17 μCi of [methyl-^{3}H]thymidine (specific activity, 5 mCi/mmol) plus 10^{-5} M unlabeled thymidine. CH1cisR cells (^{14}C-labeled) were treated with 10 μM of compounds **1′a**, **1′b**, cisplatin, and carboplatin for several periods of time. In addition, an untreated control plate was included in all experiments. Immediately after drug treatment, the drug was washed off using ice-cold phosphate-buffered saline (PBS). Test (^{14}C-labeled) cells and internal standard (^{3}H-labeled) cells were then irradiated on ice with 5 and 1 Gy, respectively, of ^{60}Co γ-rays from a 2000 Ci source (dose rate, 2 Gy/min). Approximately 10^6 cells of a 1:1 mix of test and internal standard cells was then added to duplicate 2 μm pore size 25 mm polycarbonate filters (Millipore Co.) in 5 mL ice-cold PBS. Cells were then lysed by two additions of 10 mL lysis buffer (2% sodium dodecyl sulfate, in 0.1 M glycine and 0.02 M ethylenediaminetetraacetic acid (EDTA), pH 10). In the first 10 mL, proteinase K (0.5 mg/mL; Sigma Co.) was added immediately prior to use. DNA was then eluted at pH 12 using 10 mL of 0.1 M tetra propylammonium hydroxide, containing 0.1% sodium dodecyl sulfate and 0.02 M EDTA. The elution rate was 0.010 mL/min (using a Pharmacia Biotech peristaltic pump), and fractions were collected at 90 min intervals over 24 h. The ^{14}C and ^{3}H DNA radioactivity was then determined in each fraction and from the filters by liquid scintillation counting (Wallac 1209 Rackbeta). Results are expressed as fraction ^{14}C retained vs fraction ^{3}H (internal standard). DNA ICL units/dalton × 10^9 were calculated using the expression

$$\text{ICL index} = [(1 - r_o/1 - r)^{1/2} - 1] \times P_b$$

where r and r_o are the fractions of ^{14}C-labeled DNA for treated vs control cells remaining on the filter when 60% of ^{3}H-labeled DNA is retained on the filter and P_b is the radiation-induced break probability/Da. Control experiments were carried out to test for the presence of cisplatin-induced single-strand breaks. Experiments were carried out in triplicate.

Determination of Platinum Binding to DNA in Culture Cells. Culture plates containing exponentially growing CH1cisR cells in 10 mL of Dulbecco's modified Eagle's Medium (DMEM) medium (cell density = 2 × 10^5 cells/mL) were exposed to 10 μM of compounds **1′a**, **1′b**, cisplatin, and carboplatin dissolved in DMEM. The plates were incubated for 1, 3, or 24 h under the conditions described above. Following drug incubation, the culture medium was removed from the plates and the cell plates were washed with PBS. Subsequently, the cells were lysed with 700 μL of a buffer solution containing 150 mM TrisHCl, pH 8.0, 100 mM EDTA, and 100 mM NaCl, incubated for 15 min at 4 °C, and centrifuged at 12 000 rpm for 15 min in a microfuge. Supernatants were treated for 3 h at 37 °C with 20 μg/mL of proteinase K (Boehringer). Afterward, supernatants were incubated for 16 h at 37 °C with 4100 μg/mL of RNase A (Boehringer). Finally, DNA was extracted with a volume of phenol−chloroform−isoamyl alcohol (50 + 49 + 1), precipitated with 2.5 volumes of cold ethanol and 0.1 volumes of 3 M sodium acetate, washed with 75% of ethanol, dried, and resuspended in 1 mL of water. The DNA content in each sample was measured by UV spectrophotometry at 260 nm in a Shimadzu UV-240 spectrophotometer, and the platinum bound to DNA was determined by total reflection X-ray fluorescence (TXRF). Experiments were carried out in triplicate.

Measurements of Platinum Accumulation in Culture Cells. Cultures plates containing exponentially growing CH1cisR cells in 10 mL of DMEM medium (cell density = 2 × 10^5 cells/mL) were exposed to 10 μM of compounds **1′a**, **1′b**, cisplatin, and carboplatin dissolved in DMEM medium for 1, 3, or 24 h. Cells were washed with ice-cold PBS, scraped, and resuspended in 700 μL of lysis buffer containing 20 mM TrisHCl, pH 7.5, 2 mM EDTA and 0.4% Triton X-100,

incubated at 4 °C for 15 min, and centrifuged at 12 000 rpm for 15 min in a centrifuge. Afterward, supernatants were treated for 3 h at 37 °C with 20 μg/mL of proteinase K (Boehringer). The platinum content in the samples was determined by TXRF. Experiments were carried out in triplicate.

TXRF Measurements. The analysis by TXRF was performed using a Seifert Extra-II spectrometer (Seifert, Ahrensburg, Germany). TXRF determinations were carried out according to a procedure previously reported.[39] Briefly, a 100 μL sample of either cell supernatants or cellular DNAs from the CH1cisR cell cultures was introduced in a test tube of 2 mL. This solution was standardized with 100 ng/mL of Vanadium (Merck (Darmstadt,Germany) ICP Vanadium standard solution). Afterward, the sample was introduced into a high-purity nitrogen flow concentrator at a temperature of 70 °C until the volume was reduced 5 times. An aliquot of 5 μL was then taken, deposited on a previously cleaned quartz-made reflector, and dried on a ceramic plate at a temperature of 50 °C. The entire process was done in a laminate flow chamber (model A-100). The samples were analyzed following the X-ray Molybdenum line under working conditions of 50 kV and 20 mA with a live time of 1000 s and a dead time of 35%. Spectra were recorded between 0 and 20 keV. Fifteen elements were simultaneously analyzed as follows: P, S, K, Ca, V, Fe, Cu, Zn, As, Br, Rb, Sr, Ni, Mn, and Pt to obtain a correct deconvolution of profiles associated with the general spectrum. The Pt line was used for Pt quantification. The analytical sensitivity of the TXRF measurements was 0.3−22.4 ng Pt in a solution volume of 100 μL, with repeatability between 2 and 8% ($n = 3$).

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Supporting Information Available: Tables listing detailed crystallographic data, atomic positional parameters, and bond lengths and angles for complexes **1a** and **1′b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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