Brief Articles

Preparation and Characterization of Novel *trans*-[$PtCl_2$(amine)(isopropylamine)] Compounds: Cytotoxic Activity and Apoptosis Induction in *ras*-Transformed Cells[§]

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The synthesis and chemical characterization of three new transplatinum complexes of structural formula *trans*-[$PtCl_2$(amine)(isopropylamine)] (amine = *n*,*n*-dimethylamine, propylamine, and butylamine), **1**−**3**, are described. Cytotoxicity tests in tumor cell lines sensitive to *cis*-DDP (Jurkat, Hela, and Vero) and also in tumor cell lines overexpressing *ras* oncogenes and resistant to *cis*-DDP (HL-60 and Pam 212-*ras*) show that complexes **1** and **3** have higher cytotoxic activity than cisplatin. Moreover, these two *trans*-Pt(II) complexes kill Pam 212-*ras* cells through apoptosis induction. These results suggest that *trans*-$PtCl_2$ complexes with asymmetric aliphatic amines may be considered a new class of biologically active *trans*-platinum drugs.

Introduction

Since the discovery of the anticancer properties of cisplatin, *cis*-diamminedichloroplatinum(II), it has been generally accepted, via structure−activity relationships, that a *cis* configuration of the two leaving groups is essential for antitumor activity of Pt(II) compounds.[1] However, several exceptions to the rule-of-thumb of two good leaving groups in the *cis* configuration necessary for antitumor activity of Pt(II) complexes have been reported in recent years. These exceptions are the following: (i) *trans*-$PtCl_2$(L)(L′) complexes with planar heterocyclic ligands as inert groups,[2] (ii) polynuclear platinum complexes with bridging diamine linkers of the composition [{*trans*-$PtCl(NH_3)_2$}$_2${μ-$NH_2(CH_2)_n$-NH_2}]$^{2+}$ (n = 2−6) or complexes containing two *trans*-$PtCl(NH_3)_2$ units linked by a $NH_2(CH_2)_6NH_2$-*trans*-$Pt(NH_3)_2$-$NH_2(CH_2)_6$-NH_2 diamine chain,[3] and (iii) *trans*-$PtCl_2$ complexes with an imino ether as the inert group.[4]

These results are of fundamental importance because these new *trans*-platinum complexes show high activity in vitro and in vivo against tumor cell lines resistant to cisplatin (*cis*-DDP, *cis*-[$PtCl_2(NH_3)_2$]),[5] and therefore these complexes may have clinically important differences in cellular and biochemical pharmacology relative to *cis*-DDP. In this way, the determinant factors of cytotoxicity may, thus, not follow the same patterns as found for cisplatin and its analogues. These differences in biochemical pharmacology may be systematically exploited to design complexes with an altered spectrum of antitumor activity and for activity in cisplatin-resistant tumors. Moreover, it has been found that the structural array [{*trans*-$PtCl(NH_3)_2$}$_2${μ-$NH_2(CH_2)_n$-NH_2}]$^{2+}$ (n = 2−6) is especially efficient in causing irreversible B → Z transitions in poly(dG-dC)poly(dG-dC).[6]

To identify new *trans*-Pt(II) complexes endowed with cytotoxic properties, we have synthesized and characterized several *trans*-[$PtCl_2$LL′] complexes, where L = isopropylamine and L′ = *n*,*n*-dimethylamine, propylamine, or butylamine. The results reported in this paper show that compounds *trans*-[$PtCl_2$(*n*,*n*-dimethylamine)-(isopropylamine)], compound **1**, and *trans*-[$PtCl_2$(butylamine)(isopropylamine)], compound **3**, have higher cytotoxic activity than *cis*-DDP in cell lines sensitive to this drug (Jurkat, HeLa, and Vero). Moreover, compounds **1** and **3** circumvent *cis*-DDP resistance in tumor cells overexpressing *ras* oncogenes (HL-60 and Pam 212-*ras*). Interestingly, these two novel *trans*-Pt(II) complexes kill Pam 212-*ras* cisplatin-resistant cells through apoptosis induction.

Results and Discussion

Synthesis and Characterization of the *trans*-[$PtCl_2$(amine)(isopropylamine)] Compounds. The reactions between *cis*-[$PtCl_2$(amine)$_2$], where amine = *n*,*n*-dimethylamine (**1**), propylamine (**2**), or butylamine (**3**), and isopropylamine in water at 70 °C and subsequent addition of hydrochloric acid afforded the corresponding compounds (Scheme 1) that were characterized by elemental analyses, infrared spectra, and ^{1}H and ^{13}C NMR spectra in $CDCl_3$ as solvent. The microanalytical data are consistent with the empirical formulas

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§ Abbreviations: *cis*-DDP, cisplatin, *cis*-diamminedichloroplatinum-(II); *trans*-DDP, transplatin, *trans*-diamminedichloroplatinum(II); DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate; PBS, phosphate-buffered saline; PI, propidium iodide; SD, standard deviation; SDS, sodium dodecyl sulfate; TMS, tetramethylsilane; Tris, tris-(hydroxymethylamino)methane; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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Scheme 1. Synthesis of the *trans*-[$PtCl_2$(amine)-(isopropylamine)] Compounds

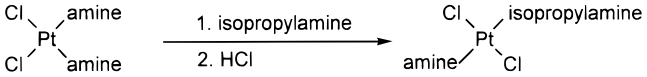
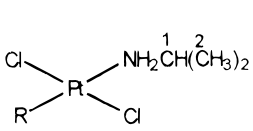


Table 1. ^{1}H NMR Parameters δ (ppm) of the Amines and Their Complexes[a]



1, R = $NH(CH_3)_2$ (1')

2, R = $NH_2CH_2CH_2CH_3$ (1' 2' 3')

3, R = $NH_2CH_2CH_2CH_2CH_3$ (1' 2' 3' 4')

	isopropylamine	**1**	**2**	**3**
H1	2.95 sp 1H *J* (6.3)	3.29 s 1H *J* (6.4)	3.29 m 1H	3.35 m 1H
H2	0.89 d 6H *J* (6.3)	1.33 d 6H *J* (6.3)	1.33 d 6H *J* (6.4)	1.33 d 6H *J* (6.1)
NH_2	0.95 sbr 2H	3.17 sbr 2H	3.36 sbr 2H	3.35 sbr 2H
H1′		2.67 d 6H *J* (6.0; 23.7[b])	2.63 t 2H *J* (7.2)	2.67 t 2H *J* (6.8)
H2′			1.43 sx 2H *J* (7.2)	1.37 m 2H
H3′			0.89 t 3H *J* (7.2)	1.37 m 2H
H4′				0.89 t 3H *J* (7.1)
NH_2′			1.13 s 2H	1.14 sbr 2H
NH′		3.85 sbr 1H		

[a] The numbers in parentheses correspond to $J(^1H-^1H)$ in Hz; sbr, singlet broadened; s, singlet; d, doublet; t, triplet; sx, sextet; sp, septet; m, multiplet. [b] $J(^{195}Pt-^1H)$, solvent DMSO.

$C_5H_{16}N_2PtCl_2$ (**1**), $C_6H_{18}N_2PtCl_2$ (**2**), and $C_7H_{20}N_2PtCl_2$ (**3**) which indicate the structure [$PtCl_2$(amine)(isopropylamine)]. The assignment of the *trans* geometry is supported after IR spectral data (single bands at 332, 333, and 343 cm^{-1} in compounds **1**, **2**, and **3**, respectively) which suggest *trans*-chloride ligands. The IR spectra also show bands at 433, 441, and 448 cm^{-1}, respectively, assigned to ν asymmetric Pt−N.

The ^{1}H and ^{13}C NMR data confirm the structures of the complexes proposed by IR data. The proton NMR spectra are well-defined allowing the assignment of all the signals and the integration of the proton signal according to the requirements. The ^{1}H NMR data for compounds **1**−**3** and isopropylamine are shown in Table 1. The signal corresponding to the amine group of isopropylamine appears strongly deshielded, in the complexes ($\Delta\delta$ = 2.22−3.79 ppm), and integrates as two protons. The aliphatic protons are also shifted to low field with respect to the free amine. Similarly, the ^{13}C NMR show the same effect, so the deshielding is $\Delta\delta$ = 1.8−2.1 ppm for C1 of isopropylamine and 4.2−8.6 for C1 of amine.

Cytotoxic Activity and Apoptosis Induction of the Synthesized *trans*-Pt(II) Compounds. In contrast with the standard structure−activity relationships for Pt(II) compounds, it has been lately shown that some new *trans*-Pt(II) compounds are endowed with important antitumor properties.[7] In view of these recent findings, we have tested the cytotoxic activity of the synthesized *trans*-[$PtCl_2$(amine)(isopropylamine)] complexes in cell lines sensitive and resistant to *cis*-DDP. The results shown in Table 2 indicate that in general the *trans*-[$PtCl_2$(amine)(isopropylamine)] compounds exhibit a cytotoxic activity in the same micromolar range as *cis*-DDP. However, compound **2** displays a cytotoxic activity in Hela and Vero cells lower than that of *cis*-DDP. It is interesting to note that compounds **1**−**3** show remarkable cytotoxic activity in *cis*-DDP-resistant cells overexpressing *ras* oncogenes (HL-60 and Pam 212-*ras*). In particular, compounds **1**−**3** have IC_{50} values 1.5-, 1.25-, and 1.4-fold lower than *cis*-DDP in HL-60 cells which are tumor cells overexpressing c-*myc* and N-*ras* oncogenes.[8] Moreover, compounds **1**−**3** exhibit against Pam 212-*ras* cells transformed by the H-*ras* oncogene[9] IC_{50} values 26-, 5-, and 7-fold lower than *cis*-DDP, respectively. In addition, these novel *trans*-Pt compounds display a good "in vitro" therapeutic index (T.I.) since they have a much lower toxicity in normal Pam 212 cells than in Pam 212-*ras* cells (T.I. of 9.3, 1.6, and 3.4, respectively, versus 0.7 for *cis*-DDP). On the other hand, from the analysis of the cytotoxicity curves of the synthesized *trans*-Pt(II) compounds in Pam 212-*ras* cells, it may be concluded that compound **1** is the more active one since at a concentration of 50 μM it kills nearly 100% of the cells while compounds **2** and **3** kill 60% and 70% of the cells, respectively (data not shown). Moreover, it is interesting to note that even at a concentration higher than 200 μM, *cis*-DDP, *cis*-[$PtCl_2$-(isopropylamine)$_2$], and *trans*-DDP are unable to produce complete cell killing. Altogether, the cytotoxicity data presented here indicate that *trans*-$PtCl_2$ compounds with asymmetric aliphatic amines exhibit specific cytotoxic properties different from those of *cis*- and *trans*-DDP compounds. We think that these findings indicate that *trans*-$PtCl_2$ compounds with asymmetric aliphatic amines constitute a new class of cytotoxic *trans*-platinum complexes. It should be pointed out that, so far, it was only known that *trans*-$PtCl_2$ compounds with the same aliphatic amine in the two inert groups had poor activity.[1]

Table 2. ^{13}C NMR Parameters δ (ppm) of the Amines and Their Complexes

	isopropylamine	**1**	**2**	**3**
C1	43.0	48.7	48.5	48.4
C2	26.1	24.0	24.3	24.0
C1′		43.3	48.4	46.5
C2′			23.9	33.5
C3′			10.8	19.9
C4′				13.5

Apoptosis Induction by the Synthesized *trans*-Pt(II) Compounds in Pam 212-*ras* Cells. It has been reported that the ability of certain antitumor drugs to achieve a significant therapeutic index differentiating malignant from normal cells may be associated with tumor cell killing through apoptosis at drug concentrations significantly lower than those needed to kill normal cells.[10] On these grounds, we have analyzed whether the high cytotoxic activity of the synthesized compounds in Pam 212-*ras* cells was associated with apoptosis induction. Thus, genomic DNA from Pam 212-*ras* cells exposed during 24 h to the IC_{50} of the *trans*-Pt(II) synthesized compounds was extracted and analyzed by agarose gel electrophoresis. It may be observed in Figure 1 that compounds **1** and **3** (lanes 6 and 7, respectively) produce a strong "DNA laddering" indicative of apoptosis. In contrast, the rest of the compounds except *trans*-DDP produce a DNA smear (Figure 1, lanes 4, 5, and 8). Treatment of the Pam 212-*ras* cells with

Table 3. IC_{50} Mean Values Obtained for *trans*-[$PtCl_2$(amine)(isopropylamine)] Complexes Against Several Tumor and Normal Cell Lines[a]

	IC_{50} (μM) ± SD, cell lines					
	HL-60	JURKAT	HeLa	VERO	PAM 212	PAM 212-*ras*
1	17 ± 2.0	5 ± 0.3	32 ± 3.0	47 ± 4.0	56 ± 2.0	6 ± 0.5
2	20 ± 2.0	5 ± 0.4	43 ± 3.0	52 ± 4.0	50 ± 3.0	32 ± 1.0
3	18 ± 3.0	7 ± 0.2	35 ± 2.0	46 ± 3.0	71 ± 4.0	21 ± 2.0
trans-DDP	50 ± 3.0	22 ± 0.7	89 ± 4.0	148 ± 6.0	123 ± 5.0	164 ± 8.0
cipa	30 ± 2.0	9 ± 1.0	65 ± 2.0	93 ± 6.0	112 ± 6.0	158 ± 4.0
cis-DDP	25 ± 1.0	7 ± 0.3	38 ± 1.0	50 ± 3.0	114 ± 3.0	156 ± 6.0

[a] cipa, *cis*-[$PtCl_2$(isopropylamine)$_2$]; SD, standard deviation.

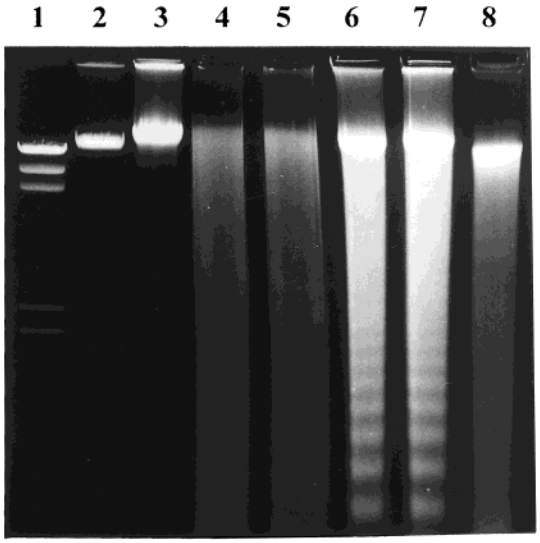


Figure 1. Agarose gel electrophoresis of DNA extracted from control Pam 212-*ras* cells (lane 2) and Pam 212-*ras* cells treated with the IC_{50} of *trans*-DDP (lane 3), *cis*-DDP (lane 4), compound **2** (lane 5), compound **1** (lane 6), compound **3** (lane 7), and compound **2** (lane 8). Lane 1: *Hin*dIII digested λ phage DNA.

the IC_{50} of *trans*-DDP (Figure 1, lane 3) only slightly alters the band of genomic DNA as expected from the well-known fact that *trans*-DDP has poor cytotoxic activity being, therefore, biologically inactive.[11] On the other hand, it was observed that the induction of apoptosis is specific of the *trans*-Pt arrangement and not due to the amine ligands coordinated to the *trans*-Pt(II) center (data not shown).

Apoptosis was also examined by phase contrast microscopy of Pam 212-*ras* cells treated with the IC_{50} of compounds **1**, **3**, and *cis*-DDP (Figure 2). It may be observed that the cells treated with compounds **1** and **3** are detached from the plate surface having a rounded morphology, both facts being indicative of apoptosis induction (Figure 2C,D, respectively). However, cells treated with *cis*-DDP are attached to the plate and do not show these alterations in cell morphology (Figure 2B).

Apoptosis induction was confirmed by fluorescence microscopy of cells stained with propidium iodide after drug treatment. Figure 3 shows the formation of apoptotic bodies in cells treated with the IC_{50} of compounds **1** and **3** (Figure 3C,D, respectively), while in cells treated with the IC_{50} of *cis*-DDP there is not evidence of apoptotic bodies since staining with PI is homogeneously distributed within the cell as an indication of death by necrosis (Figure 3B).

Quantification of apoptosis was performed by cell counting after staining with propidium iodide and

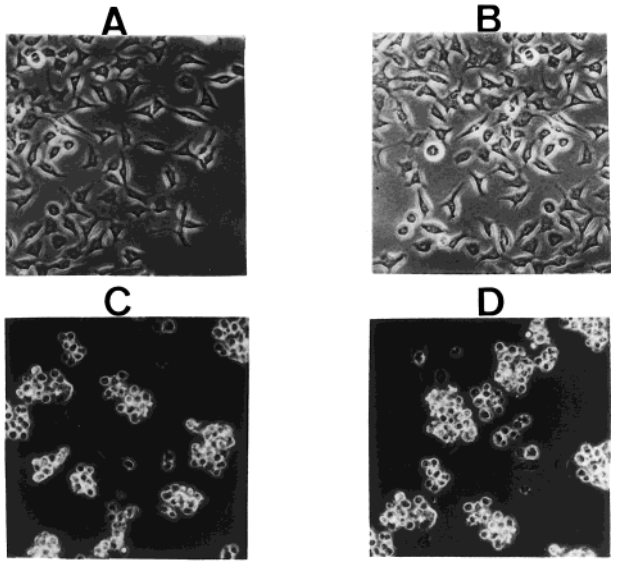


Figure 2. Morphological changes observed in untreated Pam 212-*ras* cells (A) and Pam 212-*ras* cells treated with the IC_{50} of *cis*-DDP (B), compound **1** (C), and compound **3** (D) for 10 h and photographed under a phase contrast microscope at a magnification of ×200 (reduced 75% for publication). The results shown reproduce the observations of quadruplicate independent experiments.

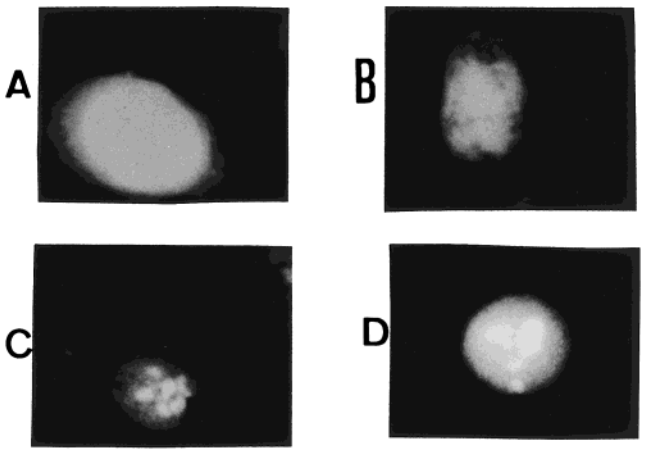


Figure 3. Fluorescence microscopy of control Pam 212-*ras* cells (A) and Pam 212-*ras* cells after treatment for 24 h with the IC_{50} of *cis*-DDP (B), compound **1** (C), and compound **3** (D). The results shown reproduce the observations of quadruplicate independent experiments.

visualization by fluorescence microscopy of Pam *ras* cells treated with the IC_{50} of the compounds for several periods of time. The results indicated that after 3 h of incubation with the IC_{50} of compounds **1** and **3**, 40% and 30% of apoptotic cells are produced, respectively, with 75% of the cells becoming apoptotic after 24−28 h. The

percentage of apoptotic cells decreased after 28 h of treatment, being 7% after 96 h. In contrast, treatment of Pam *ras* cells with *cis*-DDP did not induce apoptotic cells at any of the periods of time tested (data not shown). Altogether the apoptosis data suggest that in Pam *ras* cells the higher cytotoxic activity of compounds **1** and **3** relative to *cis*-DDP may be due to a specific induction of apoptosis at low drug concentration. To our knowledge compounds **1** and **3** are the first reported *trans*-$PtCl_2$ complexes with asymmetric aliphatic amines able to induce apoptosis in cisplatin-resistant cells. However, apoptosis induction has also been observed after treatment of CH1 ovarian carcinoma cells sensitive to cisplatin with JM335, the *trans* analogue of ammine(cyclohexylamine)dihydroxodichloroplatinum(IV).[12]

Conclusion

The biochemical mechanism(s) whereby *ras* oncogenes impart *cis*-DDP resistance are still obscure.[11] However, there is mounting evidence indicating that *ras* oncogenes mainly affect the expression of genes involved in repairing DNA damage produced by *cis*-DDP. In fact, it has been found that the DNA in MCF-7-*ras* cells treated with *cis*-DDP under induced conditions is intact, whereas extensive degradation of DNA occurs in similarly treated cells under noninduced conditions suggesting that the *ras* gene product may play a role in the DNA repair process.[13] However, it should be pointed out that not all cells and especially human cells bearing *ras* mutations show a change in cisplatin sensitivity.[14,15] Since *ras* oncogenes may be involved in DNA repair, it is likely that these novel *trans*-Pt complexes form DNA adducts capable to circumvent *cis*-DDP resistance. In fact, preliminary results indicate that compound **1** induces a higher amount of DNA interstrand cross-links than *cis*-DDP. Moreover, this novel *trans*-Pt compound binds to alternating purine−pyrimidine sequences (laboratory data).

In summary, the results presented in this paper indicate that coupling to a *trans*-$PtCl_2$ center of asymmetric aliphatic amines may result in novel *trans*-platinum compounds with cytotoxic properties different from those of *cis*- and *trans*-DDP.

Experimental Section

The infrared spectra were recorded in Nujol mulls and KBr pellets in the 4000−200 cm^{-1} range using a Perkin-Elmer model 283 spectrophotometer. NMR spectra were recorded on a Bruker AMX-300 spectrometer in $CDCl_3$ with TMS as internal standard. The C, H, and N analyses were carried out with a Perkin-Elmer 2400 microanalyzer. The mass spectra were recorded in a V.G. AUTODPEC high-resolution spectrometer, using *m*-NBA or H_2SO_4 as a matrix, depending on the compound. The analytical data were within the limits of experimental error (±0.4%).

All solvents were purified by standard methods prior to use. Platinum(II) salts were a gift from Johnson Matthey plc. The amine ligands were purchased from Aldrich.

Synthesis of the *trans*-[$PtCl_2$(amine)(isopropylamine)] Compounds 1−3. The synthesis of *trans*-Pt(II) complexes was carried out using the difference in the *trans* effect of halide and amine ligands in platinum(II) complexes to achieve selective substitution and thus control of stereochemistry. Variations in the experimental conditions established for *trans*-[$PtCl_2(NH_3)_2$] are required.[16] In all cases, the initial compound was the one that contained $PtCl_2$(amine)$_2$, where amine is the amine different from the isopropylamine.

***trans*-[$PtCl_2$(dimethylamine)(isopropylamine)], 1.** A suspension of *cis*-[$PtCl_2$(dimethylamine)$_2$] (1.0 g, 2.7 mmol) in water (10 mL) was treated with 4 equiv of isopropylamine (0.6 g, 10.8 mmol). The mixture was stirred and heated at 70 °C until a clear pale yellow solution was obtained and then brought to reflux. After the solution was allowed to cool at room temperature, hydrochloric acid (12 M, 2.9 mL) was added and the solution heated to reflux for 6 h. After cooling in an ice bath, the product was collected by filtration and washed with water (yield 64%): IR 332 cm^{-1} (Pt−Cl); FAB-MS 370.18.

The following compounds were obtained in a similar way with the indicated variations.

***trans*-[$PtCl_2$(isopropylamine)(propylamine)], 2.** Once a clear pale yellow solution was obtained it was allowed to cool at room temperature. Then it was filtered off and the filtrate was dried by evaporation. Subsequently, it was dissolved by the addition of water. Finally, hydrochloric acid (12 M) was added and the solution heated to reflux for 24 h (yield 60%): IR 333 cm^{-1} (Pt−Cl); FAB-MS 384.21.

***trans*-[$PtCl_2$(butylamine)(isopropylamine)], 3.** Compound **3** was synthesized as indicated above for compound **1** (yield 55%): IR 343 cm^{-1} (Pt−Cl); FAB-MS 398.24.

Biological Methods. Reagents and compounds: The 100-mm culture and microwell plates were obtained from NUNCLON (Roskilde, Denmark). MTT was purchased by Sigma Chemical Co.; FCS was supplied by GIBCO-BR; *cis*-DDP, *cis*-diamminedichloroplatinum(II), and *trans*-DDP, *trans*-diamminedichloroplatinum(II), were purchased from Sigma Chemical Co. Stock solutions of the compounds at a concentration of 1 mg/mL in PBS were freshly prepared before use.

Cell lines and culture conditions: HL-60 (human promyelocytic leukemia line with activation of c-*myc* and N-*ras* oncogene),[8] Jurkat (human acute T-cell leukemia line), and HeLa (human cervix epithelial carcinoma line) cells were cultured as described previously.[19] Culture and characterization of Pam 212 (normal murine keratinocytes) and Pam 212-*ras* (murine keratinocytes transformed with the H-*ras* oncogene and resistant to *cis*-DDP) cells have been reported elsewhere.[9,17]

Drug cytotoxicity: Cell survival in compound-treated cultures was evaluated by using the MTT method.[18] Compounds were added to microwells containing the cell cultures at final concentrations of 0−200 μM; 24 h later, cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker microplate reader 2001. IC_{50} values were calculated from curves constructed by plotting cell survival (%) versus compound concentration (μM). All experiments were made in quadruplicate.

"DNA laddering" assay: Pam 212-*ras* cells (5×10^5 cells/mL) were plated in P100 sterile plates. The cells were treated with the IC_{50} of the compounds for 24 h under the above-mentioned conditions. Evaluation of genomic DNA laddering was performed as described previously.[19]

Changes in cell morphology: Examination of cell morphology associated with apoptosis in untreated and treated Pam 212-*ras* cells was carried out as described previously.[10]

Detection of apoptotic nuclei: Pam 212-*ras* cells (10^5 cells/plate) were treated with equitoxic concentrations of the compounds (IC_{50}) for treatment periods of 1, 3, 10, 24, 28, 48, 72, and 96 h. Staining of nuclei was carried out as previously reported.[19,20] Apoptotic nuclei were easily distinguished from normal nuclei because they were condensed, brightly fluorescent, and often fragmented.

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