DNA Interstrand Cross-Linking Efficiency and Cytotoxic Activity of Novel Cadmium(II) – Thiocarbodiazone Complexes

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*We have prepared mono- and binuclear complexes of Zn^{II} and Cd^{II} with bis(2-pyridyl aldehyde) thiocarbodiazone (H_2L^1) and bis(methyl 3-pyridyl ketone) thiocarbodiazone (H_2L^2). Cytotoxicity data against the ovarian tumor cell line A2780cisR (acquired resistance to cisplatin) indicate that the mononuclear complex Cd/H_2L^2 (**1**) and the binuclear complex Cd_2/H_2L^1 (**4**) are able to circumvent cisplatin resistance and that their cytotoxic activity does not substantially vary after depletion of intracellular levels of glutathione. Moreover, DNA binding studies show that complexes **1** and **4** have higher efficiency than cisplatin at forming DNA interstrand cross-links in both naked pBR322 plasmid and A2780cisR cellular DNA. Interestingly, the thiocarbodiazone ligands alone do not show the biological properties of complexes **1** and **4**. Altogether these results suggest that DNA interstrand cross-link formation by compounds **1** and **4** might be related with their cytotoxic activity in cisplatin-resistant cells. We think that compounds **1** and **4** may represent a novel structural lead for the development of cadmium cytotoxic agents capable of improving antitumor activity in cisplatin-resistant tumors.*

KEYWORDS:

antitumor agents · cadmium · cisplatin · DNA damage · thiocarbodiazones

Introduction

It is well-known that drug resistance represents the major limitation for the success of antitumor drugs such as cisplatin (*cis*-diamminedichloroplatinum(II); *cis*-DDP) in the treatment of cancer. On the other hand, glutathione (GSH) has been implicated in cisplatin resistance by reducing drug accumulation through the multidrug resistance associated protein (MRP),[1] by reacting with the *cis*-Pt^{II} center to form inactive species,[2] and by enhancing DNA repair.[3] In addition, several studies have shown a good correlation between GSH levels and sensitivity to cisplatin and other platinum compounds,[4, 5] and between glutathione-S-transferase activity and the clinical response to cisplatin in head and neck cancers.[6] Therefore, it is likely that cisplatin resistance can be circumvented by generating platinum and other metal containing drugs that exhibit low reactivity toward glutathione.

It has been previously reported that metallic complexes of thiocarbodiazone derivatives may have antibacterial, antiviral, and antitumor properties.[7, 8] Moreover, we have recently shown that thiosemicarbazone ligands coupled to Zn^{II} and Cd^{II} metal centers may overcome cisplatin resistance in murine keratinocytes overexpressing the H-*ras* oncogene.[9] In view of these encouraging results we have extended our study to four novel Zn^{II} and Cd^{II} complexes with bis(2-pyridyl aldehyde) thiocarbodiazone (H_2L^1) and bis(methyl 3-pyridyl ketone) thiocarbodiazone (H_2L^2) as ligands, namely Cd/H_2L^2 (**1**), Zn/H_2L^2 (**2**), Zn_2/H_2L^1 (**3**), and Cd_2/H_2L^1 (**4**). The results reported here show that the mono- and binuclear Cd^{II} – thiocarbodiazone complexes **1** and **4** are able to overcome cisplatin resistance in A2780cisR human ovarian tumor cells which contain high levels of glutathione. Interestingly, the cytotoxic activity of **1** and **4** is not significantly altered by previous cell treatment with L-buthionine sulfoximine (L-BSO), which decreases the intracellular levels of GSH (glutathione). In vitro and in vivo evaluation of the formation of DNA interstrand cross-links (ISCs) by these novel Cd^{II} – thiocarbodiazone complexes indicates that **1** and **4** produce an increase in DNA ISCs with increasing drug concentrations.

Results

Polydentate diprotic Schiff-base ligands H_2L^1 and H_2L^2 are able to form mono- and binuclear complexes.[10] When H_2L^1 was treated with MCl_2 (M = Zn or Cd) the resulting products $[M_2L^1Cl_2]$ were

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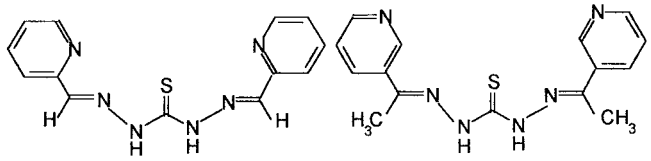

H_2L^1 H_2L^2

formulated with a doubly deprotonated ligand to bind two metal ions, while when H_2L^2 was treated with the same salts only monometallic complexes $[M(H_2L^2)Cl_2]$ were obtained.

The most significant changes in the infrared spectra of the ligands upon complexation are the presence of a new band near 3450 cm^{-1} in the spectrum of complex **1**, which is assigned to the OH stretching frequency from water molecules, the increase of $\tilde{\nu}$ for the C=N band, and the shift of the C=S (thioamide IV) band in all complexes.[11] These data indicate the participation of the thioamide group in the coordination to the metal ions.

Cytotoxic activity of the thiocarbodiazone – metal complexes

We have tested the cytotoxic activity of complexes **1** – **4**, the thiocarbodiazone ligands, and the antitumor drug *cis*-DDP against A2780 and A2780cisR cells after treatment periods of 24 and 96 hours (Table 1). Table 1 A shows that in A2780 cells and for a drug-treatment period of 24 hours **1** and **4** displayed IC_{50} values in a μM range similar to that of *cis*-DDP. Interestingly, however, complexes **1** and **4** were about 18 and 50 times more active, respectively, than *cis*-DDP in the *cis*-DDP-resistant cell line A2780cisR (IC_{50} values of 7.0, 2.5, and 125 μM, respectively). Moreover, **1** and **4** had a much better resistance factor (defined as IC_{50} in A2780cisR cells/IC_{50} in A2780 cells) than *cis*-DDP (resistance factors of 0.50, 0.25, and 15.60, respectively). Similar data were obtained for a drug-treatment period of 96 hours but the cytotoxic activity observed was on average three to four times higher than that obtained after a 24-hour period (Table 1 B). In addition, the data of Table 1 show that the Zn^{II} – thiocarbodiazone complexes **2** and **3** and the thiocarbodiazone ligands H_2L^1 and H_2L^2 exhibited poor cytotoxic activity in the cell lines tested.

As GSH is involved in intracellular detoxification of metal drugs,[12] we have also evaluated the effect of GSH on the cytotoxic activity of complexes **1** – **4**, the thiocarbodiazone ligands, and *cis*-DDP by using L-BSO to decrease the levels of GSH in A2780cisR cells prior to drug treatment. Our determinations of GSH intracellular content indicated that the A2780cisR cell line possesses glutathione levels about six times higher than the parental A2780 cell line (GSH levels: A2780: 8.5 (±0.5); A2780cisR: 50 (±0.8) nmol per mg of protein; $p < 0.01$). These data are in agreement with previous data reported in the literature.[12] Table 1 shows that while potentation of cytotoxicity was negligible for Cd^{II} – thiocarbodiazone complexes **1** and **4** in both A2780 and A2780cisR cells (no significant difference was observed to the control values), cytotoxicity was significantly higher for *cis*-DDP in A2780cisR cells ($p < 0.01$).

Table 1. *IC_{50} mean values obtained for the synthesized complexes **1** – **4**, the thiocarbodiazone ligands, and* cis*-DDP against A2780 and A2780cisR cell lines after 24- or 96-hour drug-treatment periods (A and B, respectively).*

(A)	$IC_{50} \pm SD$ [μM][a] Cell line			
Test compound	A2780	A2780+L-BSO[b]	A2780cisR	A2780cisR+L-BSO[b]
1	15 ± 2	12 ± 2	7.0 ± 0.5	6.5 ± 0.1
2	> 200	> 200	> 200	> 200
3	> 200	> 200	> 200	> 200
4	10 ± 2	8 ± 2	2.5 ± 0.3	2.4 ± 0.4
cis-DDP	8 ± 1	7 ± 1	125 ± 10	50 ± 3
H_2L^1	> 200	> 200	> 200	> 200
H_2L^2	> 200	> 200	> 200	> 200
(B)	$IC_{50} \pm SD$ [μM][a] Cell line			
Test compound	A2780	A2780+L-BSO[b]	A2780cisR	A2780cisR+L-BSO[b]
1	5.0 ± 0.2	4.0 ± 0.5	2.5 ± 0.5	2.3 ± 0.7
2	> 100	> 100	> 100	> 100
3	> 100	> 100	> 100	> 100
4	3.0 ± 0.6	2.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.2
cis-DDP	4.0 ± 0.3	3.8 ± 0.5	70 ± 5	22 ± 2
H_2L^1	> 100	> 100	> 100	> 100
H_2L^2	> 100	> 100	> 100	> 100

[a] SD = standard deviation. [b] +L-BSO indicates that the cells were preincubated for 24 hours with 50 μM of L-buthionine sulfoximine in order to deplete cellular glutathione levels.

In vitro DNA interstrand cross-link formation

As the cytotoxic activity of **1** and **4** might be related with their DNA binding mode and, on the other hand, *cis*-DDP induces low levels of DNA interstrand cross-linking,[13] we have analyzed the ability of **1** – **4** to form this type of adduct in the linear pBR322 plasmid. Figure 1 shows the results of the interstrand cross-linking assay for **1** – **4**. As expected, the control native pBR322 plasmid migrates as a DNA band corresponding to double-stranded DNA and the control denatured pBR322 plasmid migrates as a DNA of higher mobility corresponding to single-stranded DNA (lanes 1 and 2, respectively). It may be also observed that all pBR322 plasmid migrate as double-stranded DNA forms after incubation with complexes **1** and **4** at 10 and 100 μM drug concentrations (lanes 7 – 10). These data indicate that **1** and **4** are forming cross-links between pBR322 DNA complementary strands. In contrast, complexes **2** and **3** do not appear to form ISCs because, at 100 μM drug concentration, all plasmid DNA migrates as a single-stranded DNA band (lanes 3 and 4, respectively). In addition, Figure 1 also shows that *cis*-DDP forms DNA ISCs at a 100 μM drug concentration (lane 6) but not at 10 μM drug concentration (lane 5) since, at this lower *cis*-DDP concentration, the band corresponding to double-stranded DNA is not detected (lane 5). Densitometric analysis of the DNA bands indicated that at 100 μM drug concentration the amount of double-stranded DNA formed with **1** and **4** is twofold higher than with *cis*-DDP. These data indicate that the capacity of **1** and **4** to form DNA ISCs is at least twofold higher than that of *cis*-DDP.

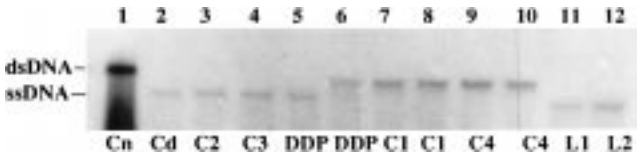

Figure 1. *Patterns of single- and double-stranded DNA after melting of the linear pBR322 plasmid DNA which was incubated for 24 hours at 37 °C in 10 mM $NaClO_4$ with: lane 1, native double-stranded DNA (Cn); lane 2, denatured single-stranded DNA (Cd); lane 3, complex **2** (C2; 100 μM); lane 4, complex **3** (C3; 100 μM); lanes 5 and 6,* cis*-DDP (DDP; 10 and 100 μM, respectively); lanes 7 and 8, complex **1** (C1; 10 and 100 μM, respectively); lanes 9 and 10, complex **4** (C4; 10 and 100 μM, respectively); lane 11, H_2L^1 (L1; 100 μM); and lane 12, H_2L^2 (L2; 100 μM).*

In vivo DNA interstrand cross-link formation

In vivo DNA interstrand cross-linking efficiency was quantified by exposing A2780cisR cells to **1** and **4** for 24 hours and determining levels of DNA ISCs. *cis*-DDP was used as a positive control and complex **3** as a negative one for ISC formation. Figure 2 shows that **3** did not produce detectable levels of ISCs at concentrations up to 100 μM. In contrast, complexes **1** and **4** produced increasing ISCs with increasing drug concentrations. At an equimolar concentration of 100 μM, ISCs were 1.4 times higher for **1** and twice as high for **4**, relative to levels induced by *cis*-DDP.

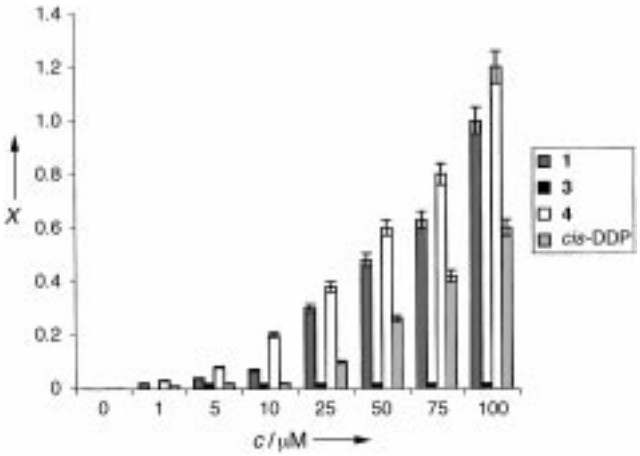

Figure 2. *Formation of DNA interstrand cross-links in A2780cisR cells as measured by alkaline filter elution immediately after 24 h exposure to complexes **1**, **3**, **4**, or* cis*-DDP. X = Cross-link index (see Experimental Section).*

Discussion

We report herein evidence to indicate that the cytotoxic activity of novel mono- and binuclear Cd^{II} – thiocarbodiazone complexes may be related with their efficiency of DNA interstrand cross-linking in cisplatin-resistant A3780cisR cells.

The cytotoxicity data indicate that complexes **1** and **4** are approximately 18 and 50 times more potent, respectively, than *cis*-DDP in A2780cisR cells. The cytotoxicity results obtained in the presence or absence of preexposure to L-BSO (in cells having high intrinsic GSH levels) suggest that **1** and **4** are less susceptible to reactivity with intracellular thiol-containing species than *cis*-DDP. In fact, previous findings have revealed an increase in cisplatin cytotoxicity by depleting cellular GSH levels.[14] So, it is likely that complexes **1** and **4** show reduced preferential reaction with soft nucleophiles, such as GSH, over hard nucleophiles such as the DNA bases.

The cytotoxic activity of complexes **1** and **4** might be related with their DNA interstrand cross-linking efficiency; **1** and **4** are potent cytotoxic agents in A2780cisR cells, and in these tumor cells they form higher levels of ISCs than *cis*-DDP. The formation of high levels of ISCs has also been observed for other transition metals, for instance, in the reaction of Cr^{VI} with DNA. However, in this case reduction of Cr^{VI} to Cr^{III} by ascorbate is prerequisite for the formation of DNA ISCs.[15] The higher cytotoxicity and DNA interstrand cross-linking efficiency of **4** relative to **1** might be due to the fact that complex **4** is a binuclear metallic compound which contains two Cd^{II} centers. In fact, it has been previously reported that, in general, bisplatinum complexes form more DNA ISCs than their mononuclear counterparts.[16] On the other hand, the DNA sequence specificity of ISC formation by Cd^{II} metallic centers might be different from that of Pt^{II} centers; sequence-specific Pt – DNA ISC repair is probably involved in the resistance of A2780cisR cells and other cisplatin-resistant cells to a great variety of platinum complexes.[17, 18]

We think that the higher biological activity of Cd^{II} complexes relative to the Zn^{II} complexes may be a consequence of the chemical differences between Cd^{II} and Zn^{II}. Cd^{II}, as Pt^{II}, is a soft metal ion and binds avidly to soft bases such as the donor nitrogens of the DNA bases.[19] However, Zn^{II} is a harder acid and tends to bond to harder bases, such as the donor nitrogen atoms of imidazole groups. Alternatively, because Cd^{II} is a soft metal ion it could bind more strongly to the N atom of the pyridine in H_2L^1 ligand than the Zn^{II} ion could. So, the Cd^{II} – thiocarbodiazone complexes would have high stability during the DNA binding and in vitro screening conditions. In fact, many inorganic and complex zinc compounds have been screened as cytotoxic agents[20] but no promising results were obtained for most of them, except for some Zn^{II} – thiosemicarbazone complexes.[9] In contrast, tumor-cell growth inhibition has been established for several Cd^{II} complexes.[21]

The cytotoxic properties of the Cd^{II} – thiocarbodiazone complexes reported here suggest that these agents might have interesting antitumor properties, particularly in view of the fact that they are capable of circumventing *cis*-DDP resistance in A2780cisR cells. We think that complexes **1** and **4** might represent a starting point for the development of metal – thiocarbodiazone complexes which can overcome cisplatin resistance.

Experimental Section

Measurements: Elemental analyses were carried out on Perkin-Elmer model 2400 automatic maschine. Infrared spectra (4000 – 400 cm^{-1}; for KBr disks) were recorded on a Bomen-Michelson spectrophotometer. Electronic spectra (*N*,*N*-dimethylformamide solution) were recorded on a Unicam UV2 1000 E spectrometer. Mass spectra (EI, FAB, and MALDI-TOF) were carried out and registered by the Servicio Interdepartamental de Apoyo a la Investigación (SIDI) of the Universidad Autónoma de Madrid (UAM).

Reagents and drugs: Thiocarbohydrazide, pyridine-2-carboxaldehyde, methyl 3-pyridyl ketone, $ZnCl_2$, and $CdCl_2 \cdot 2.5\,H_2O$ were commercially available and used without further purification. 100-mm culture and microwell plates were obtained from NUNCLON (Roskilde, Denmark); MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma; FCS (fetal calf serum) was supplied by GIBCO-BRL; *cis*-DDP (*cis*-diamminedichloroplatinum(II)) was purchased from Sigma. Zn^{II} and Cd^{II} metal complexes, thiocarbodiazone ligands, and *cis*-DDP were dissolved in 10 mM $NaClO_4$. Stock solutions of the compounds at a concentration of 1 mg mL^{-1} were freshly prepared before use.

Cell lines and culture conditions: The pair of human ovarian tumor cell lines (A2780/A2780cisR) were cultured in DMEM (Dulbecco's modified eagles medium) supplemented with 10% FCS, together with 2 mM glutamine, 100 units mL^{-1} of penicillin, and 100 mg mL^{-1} of streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2.

Preparation of compounds: Bis(2-pyridyl aldehyde) thiocarbodiazone (H_2L^1) was prepared using the published procedure.[22] Bis-(methyl 3-pyridyl ketone) thiocarbodiazone (H_2L^2) was prepared using the following procedure: Methyl 3-pyridyl ketone (5 mmol) was added to thiocarbohydrazide (2 mmol) dissolved in hot ethanol (200 mL) with a few drops of glacial acetic acid. The solution was refluxed for 5 h and, on cooling, a pale yellow solid precipitated. The solid formed was filtered and washed with cold ethanol and dried under vacuum. Complexes $[Cd(H_2L^2)Cl_2] \cdot 5\,H_2O$ (**1**), $[Zn(H_2L^2)Cl_2]$ (**2**), $[Zn_2L^1Cl_2]$ (**3**), and $[Cd_2L^1Cl_2]$ (**4**) were synthesized following a general procedure: An ethanolic solution of metal chloride (Zn or Cd; 2 mmol) was added dropwise with stirring to an ethanolic solution of the appropriate ligand (1 mmol) at 50 °C. After continuous stirring for 1 h, a solid was isolated, washed with ethanol and diethyl ether, and dried under vacuum.

Chemical analyses

H_2L^2: Elemental analysis calcd for $C_{15}H_{16}N_6S$: C 57.60, H 5.15, N 26.90; found: C 57.30, H 4.85, N 27.04%; MS (EI): *m/z*: 312 [M^+]; selected IR results: $\tilde{\nu} = 3170$ (s), 3148 (s; both NH), 1621 (m; C=N), 1590 (m; CN and CC), 818 cm^{-1} (w; CS (thioamide IV band)); electronic spectrum:[23] $\lambda_{max} = 327$ nm.

$[Cd(H_2L^2)Cl_2] \cdot 5\,H_2O$ (1): Elemental analysis calcd for $C_{15}H_{26}CdCl_2N_6O_5S$: C 30.75, H 4.40, N 14.35; found: C 30.00, H 3.70, N 14.00%; MS (MALDI-TOF with ditranol matrix): *m/z*: 520.8 [$^{112}Cd(H_2L^2)^{35}Cl_2$+Na$^+$], 462.9 [$^{112}Cd(H_2L^2)^{35}Cl^+$], and 313 [$(H_2L^2)$+H$^+$]; selected IR results: $\tilde{\nu} = 3454$ (br; OH), 3302 (s; NH), 1625 (m; C=N), 1609 (m; CN and CC), 795 cm^{-1} (w; CS (thioamide IV band)); electronic spectrum: $\lambda_{max} = 357$ and 377 nm.

$[Zn(H_2L^2)Cl_2]$ (2): Elemental analysis calcd for $C_{15}H_{16}Cl_2N_6SZn$: C 40.15, H 3.55, N 18.75; found: C 39.70, H 3.45, N 19.00%; MS (FAB with *m*-nitrobenzyl alcohol matrix): *m/z*: 449.5 [$^{65}Zn(H_2L^2)^{35}Cl_2^+$], 415.0 [$^{65}Zn(H_2L^2)^{35}Cl^+$], and 313.1 [$(H_2L^2)$+H$^+$]; selected IR results: $\tilde{\nu} = 3257$ (s), 3171 (s; both NH), 1630 (m; C=N), 1612 (m; CN and CC), 810 cm^{-1} (w; CS (thioamide IV band)); electronic spectrum: $\lambda_{max} = 322$ and 364 nm.

$[Zn_2L^1Cl_2]$ (3): Elemental analysis calcd for $C_{13}H_{10}Cl_2N_6SZn_2$: C 32.25, H 2.05, N 17.35; found: C 32.70, H 2.65, N 17.35%; MS (FAB with *m*-nitrobenzyl alcohol matrix): *m/z*: 484.9 [$^{65}Zn_2(L^1)^{35}Cl_2^+$], 447.2 [$^{65}Zn_2(L^1)^{35}Cl^+$], 347.1 [$ZnL^{1+}$]; selected IR results: $\tilde{\nu} = 1617$ (m; C=N), 1594 (m; CN and CC), 745 cm^{-1} (w; CS (thioamide IV band); electronic spectrum: $\lambda_{max} = 366$ and 402 nm.

$[Cd_2L^1Cl_2]$ (4): Elemental analysis calcd for $C_{13}H_{10}Cl_2N_6SCd_2$: C 27.00, H 1.75, N 14.55; found: C 27.55, H 2.00, N 14.16%; MS (MALDI-TOF with ditranol matrix): *m/z*: 578.8 [$^{112}Cd_2(L^1)^{35}Cl_2^+$], 467.9 [$^{112}Cd(L^1)^{35}Cl_2^+$], 433.8 [$^{112}Cd(L^1)^{35}Cl^+$], and 396.8 [$^{112}Cd(L^1)^+$]; selected IR results: 1623 (m; C=N), 1593 (m; CN and CC), 743 cm^{-1} (w; CS (thioamide IV band); electronic spectrum: $\lambda_{max} = 322$ and 361 nm.

Drugs cytotoxicity: Cell proliferation was evaluated with a system based on the tetrazolium compound MTT which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically.[24] A2780 and A2780cisR cells[25] were plated in 96-well sterile plates at a density of 10^4 cells per well with 100 μL of medium and were then incubated for 3–4 h. Stock solutions of the test compounds dissolved in DMEM at final concentrations of 0–200 μM were added to the wells in a volume of 100 μL per well. After 24 or 96 h of incubation, freshly diluted MTT solution (50 μL; 1/5 in culture medium) was added to each well in a final concentration of 1 mg mL^{-1} and the plate was then further incubated for 5 h. Cell survival was evaluated by measuring the absorbance at 520 nm with a Whittaker microplate reader 2001. IC_{50} values were calculated from curves constructed by plotting cell survival [%] versus compound concentration [μM]. All experiments were performed in quadruplicate.

Depletion of GSH levels in A2780cisR cells: A2780cisR cells were preexposed to L-BSO (50 μM) for 24 h. This resulted in a reduction of approximately 80% in the GSH levels.[26] The growth inhibitory effect of cisplatin, complexes **1**–**4**, and the individual ligands was then determined using the MTT method (with either 24 or 96 h exposure). Following exposure, the test compounds were removed by aspiration, cells were washed with cold phosphate-buffered saline (PBS), and wells were refilled with growth medium.

Intracellular GSH content: Intracellular GSH levels were determined in A2780 and A2780cisR cells grown as specified for the cytotoxicity tests. Approximately 10^6 cells were seeded into P100 plates, and, after overnight incubation, the cells were washed twice with ice-cold PBS. Cellular GSH was then extracted using ice-cold sulfosalicilic acid (0.6%; 2 mL) followed by a 10 min incubation at 4 °C. The total GSH level in the extract was then determined according to the method of Griffiths.[27] Protein quantification was carried out after solubilization in 1 M sodium hydroxide (2 mL) using the Lowry assay.[28] The GSH levels were expressed as nmol per mg of protein.

In vivo quantitation of DNA interstrand cross-links: DNA ISCs were determined by alkaline filter elution using the A2780 cell line as described previously.[29] The DNA of the cells was labeled by seeding 10^6 cells in P100 tissue culture plates and growing for 24 hours in the presence of 0.03 μCi mL^{-1} [^{14}C]thymidine (specific activity = 51 mCi mmol^{-1}; 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^{-1}) as well as 10^{-5} M unlabeled thymidine. The ^{14}C-labeled A2780cisR cells were treated with cisplatin or complexes **1**–**4** for 24 hours at concentrations of 10–200 μM. In addition, an untreated control plate was included in all experiments. Immediately after drug-treatment, the drug was washed off using ice-cold PBS. The ^{14}C-labeled test cells and ^{3}H-labeled internal standard cells were then irradiated with 5 and 1 Gy, respectively, of ^{60}Co γ rays from a 2000-Ci source (dose rate = 2 Gy min^{-1}) while on ice. Approximately 10^6 cells of a 1:1 mix of test and internal standard cells were then added to duplicate 2 μm pore size 25-mm polycarbonate filters (Millipore) in 5 mL of 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 ethylenediaminetetraacetate (EDTA), pH 10). In the first 10 mL, proteinase K (0.5 mg mL^{-1}; Sigma) was added immediately prior to use. DNA was then eluted at pH 12 using 10 mL of 0.1 M tetrapropylammonium hydroxide, containing 0.1% sodium dodecyl sulfate and 0.02 M EDTA. The elution rate was 0.010 mL min^{-1} (using a Pharmacia Biotech peristaltic pump), and fractions were collected at 90-min intervals

over 24 hours. 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 the fraction of ^{14}C retained versus the fraction of ^{3}H (internal standard). DNA interstrand cross-links (units of Daltons $\times 10^9$) were calculated using the expression:[30]

$$\text{Interstrand cross-links} = [(1 - r_0)^{1/2} \times (1 - r)^{-1/2} - 1] \times P_b$$

In this equation, r and r_0 are the fractions of ^{14}C-labeled DNA for treated cells versus control cells remaining on the filter, when 60% of ^{3}H-labeled DNA is retained on the filter. P_b is the radiaton-induced break probability (in Daltons). Control experiments were carried out to test for the presence of cisplatin-induced single-strand breaks.

In vitro DNA cross-linking efficiency: DNA interstrand cross-link formation was evaluated as previously described.[16] In order to linearize the pBR322 plasmid, the DNA was digested in 150 mM NaCl with 10 units of *Bam* HI (unique restriction site in pBR322) per mg of DNA at 37 °C for 4 h. The linear double-stranded plasmid DNA was labeled at the 3′ end by incubation with 2.5 mCi of [α-^{32}P]dCTP per mg of DNA and 1.25 units of the Klenow fragment of *E. coli* DNA-polymerase I per mg of DNA for 30 min at room temperature. The reaction was stopped by heating at 70 °C for 5 min. The unincorporated radioactivity was removed by passing the reaction mixture through a Sephadex G-50 chromatography column. The labeled DNA was added to the eluted solution of labeled pBR322 DNA until a final concentration of 180 μg mL^{-1} was reached. Afterwards, the DNA (90 ng mL^{-1}) in 10 mM $NaClO_4$ was incubated with the drugs in concentrations of 100 μM or 200 μM for several different periods of time. Aliquots of 10 μL were removed and the reactions were ended by addition of an equal volume of the loading dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). The DNA was melted for 10 min at 90 °C and chilled on ice. Electrophoresis on 1.5% agarose gel was carried out under denaturing conditions at 20 V for 16 h. The gels were dried and autoradiographed. Band quantification was made using a Molecular Dynamics, Model 300[a] densitometer.

Statistical analysis: Where appropriate, the statistical significance was tested using a two-tailed Student's test.

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