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Reversion of Structure-Activity Relationships of Antitumor Platinum Complexes by Acetoxime but Not Hydroxylamine Ligands

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ABSTRACT

The presence of cis-configured exchangeable ligands has long been considered a prerequisite for antitumor activity of platinum complexes, but over the past few years, several examples violating this structure-activity relationship have been recognized. We report here on studies with the geometric isomers of [PtCl$_2$(acetoxime)$_2$], cis-[dichlorobis(acetoxime)platinum(II)] [1 (cis)] and trans-[dichlorobis(acetoxime)platinum(II)] [2 (trans)], as well as those of [PtCl$_2$(hydroxylamine)$_2$], cis-[dichlorobis(hydroxylamine)platinum(II)] [3 (cis)] and trans-[dichlorobis(hydroxylamine)platinum(II)] [4 (trans)]. We found that 2 (trans) is 16 times more cytotoxic than 1 (cis) and as cytotoxic as cisplatin in cisplatin-sensitive ovarian carcinoma cells (CH1). Moreover, 2 (trans) is 15 times more cytotoxic than either cisplatin or 1 (cis) in intrinsically cisplatin-resistant colon carcinoma cells (SW480). Thus, compound 2 (trans) represents a novel type of active platinum(II) complexes of the trans geometry, whereas the hydroxylamine-containing complexes conform to the classic structure-activity relationships. The reactivity of the compounds toward dGMP and DNA and their capacity to alter the structure of double-stranded DNA and form interstrand cross-links were studied by capillary electrophoresis and gel electrophoresis. The slow binding of 2 (trans) to dGMP ($\tau_{1/2} = 50$ h versus 8.9 h in the case of cisplatin), the low reactivity toward DNA, the comparatively small impact on DNA secondary structure, and the lack of detectable interstrand cross-linking suggest a mode of action fundamentally different from that of cisplatin. Implications of our findings for the minimal structural requirements (e.g., planarity around the nitrogen donor atom and/or ramified aliphatic moiety attached to the latter) of active trans-configured platinum complexes are discussed.

The resounding success of cisplatin in tumor therapy, in particular that for testicular cancer, has set off tremendous

efforts to produce other platinum drugs with comparable therapeutic value but devoid of its shortcomings (Wong and Giandomenico, 1999; Jakupec et al., 2003). Despite the achievements of carboplatin and oxaliplatin, the chances of effecting considerable advances with complexes following the classic structure-activity relationships seem to become gradually exhausted, forcing investigators to focus their efforts on nonclassic structures that might open up new avenues.

The classic structure-activity relationships, as inferred from cisplatin/transplatin and related complexes, implied that the presence of two monodentate or one bidentate exchangeable ligand(s) coordinated in the cis geometry is an

ABBREVIATIONS: 1 (cis), cis-[dichlorobis(acetoxime)platinum(II)]; 2 (trans), trans-[dichlorobis(acetoxime)platinum(II)]; 3 (cis), cis-[dichlorobis(hydroxylamine)platinum(II)]; 4 (trans), trans-[dichlorobis(hydroxylamine)platinum(II)]; cisplatin, cis-diamminedichloroplatinum(II); transplatin, trans-diamminedichloroplatinum(II); MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; EtBr, ethidium bromide; BGE, background electrolyte; TE, Tris/EDTA; TBE, Tris-borate/EDTA; ICL, interstrand cross-link; dsDNA, double-stranded DNA; sc, supercoiled; oc, open circular; T/C, treated control.

essential prerequisite for antitumor activity (Cleare and Hoeschele, 1973). The pharmacological inactivity of transplatin had been attributed primarily to its inability to induce those DNA adducts that predominate in the case of cisplatin (i.e., intrastrand cross-links between adjacent purine bases) with a variety of consequences, such as a different impact on DNA secondary structure, lower capacity of inhibiting replication and transcription, faster repair, and the lack of recognition by high mobility group domain proteins (Jamieson and Lippard, 1999).

These assumptions have turned out to be too simplistic, because several exceptions from what had seemed to be a rule have been recognized over the past few years and have been repeatedly reviewed (e.g., Pérez et al., 2000; Natile and Coluccia, 2004). However, no general criterion for considering a *trans* complex active has been used. Cytotoxicity higher than or at least equal to that of the corresponding *cis* isomer and/or that of cisplatin has mostly been taken as sufficient. Each of the following classes of active *trans* complexes recognized so far includes at least one representative with proved antitumor activity in an in vivo model: 1) platinum(II) complexes with aromatic *N*-heterocyclic ligands such as thiazole or quinoline (Farrell, 1996); 2) platinum(II) complexes with one or two iminoether ligands (Coluccia et al., 1995); 3) platinum(IV) complexes with one ammine and one aliphatic amine ligand (Kelland et al., 1995); 4) asymmetric platinum complexes with one branched aliphatic amine, such as isopropylamine, and another, nonbulky amine ligand (Pérez et al., 2003); and 5) cationic and neutral platinum(II) complexes with cycloaliphatic amines such as piperidine or piperazine (Najajreh et al., 2006). Platinum(II) complexes with cyclic ligands mimicking iminoethers (Intini et al., 2004) and with acetimine ligands (Boccarelli et al., 2006) have been reported as further classes, based on cytotoxicity data only.

Compounds of all these classes lack cross-resistance to cisplatin in cellular models of acquired cisplatin resistance (Farrell et al., 1992; Kelland et al., 1995; Coluccia et al., 1999; Pérez et al., 2003; Najajreh et al., 2006). Furthermore, some of these compounds display a cytotoxicity profile that barely correlates with that of cisplatin in the cell line panel of the NCI comprising cells from a wide variety of malignancies (Farrell, 1996), and some even proved to be active in in vivo models with intrinsic or acquired resistance to cisplatin (Kelland et al., 1995; Coluccia et al., 1999), raising the hope that an antineoplastic drug with a different clinical activity profile might emerge from these nonclassic platinum agents.

Altered kinetics of DNA binding compared with cisplatin and specific differences in DNA adduct patterns, such as increased numbers and variant forms of interstrand crosslinks, the formation of stable monofunctional DNA adducts, and DNA-protein cross-links, have been put forward as tentative explanations for the unexpected activity of these compounds. Apparently, however, none of these characteristics can be generalized to all active *trans* complexes, and subtle differences in adduct structure seem to result in a different cellular processing and different downstream effects, leading to the manifestation of cytotoxicity (Brabec and Kasparkova, 2005).

We report herein on a novel type of platinum(II) complexes in which the *trans* isomer, *trans*-[PtCl$_2$(acetoxime)$_2$] [**2** (*trans*)] (Fig. 1), displays a high cytotoxicity, whereas complexes of the type [PtCl$_2$(hydroxylamine)$_2$] are shown to con-

form to the classic structure-activity relationships. Data on the reactivity of these complexes toward dGMP and the impact on DNA secondary structure suggest that if compound **2** (*trans*) exerts its biological effects by targeting DNA, this interaction differs markedly from that of cisplatin. Structural considerations regarding *trans*-[PtCl$_2$(acetoxime)$_2$] contribute to the understanding of the structural requirements for active *trans*-platinum complexes in general.

Materials and Methods

Syntheses. *cis*-[Dichlorobis(acetoxime)platinum(II)] [**1** (*cis*)] was prepared from K$_2$[PtCl$_4$] and 2 equivalents acetoxime in water, whereas *trans*-[dichlorobis(acetoxime)platinum(II)] [**2** (*trans*)] was prepared via solid-state thermal isomerization of **1** (*cis*) (Kukushkin et al., 2004). Before experiments, the complexes were recrystallized twice from hot (80–90°C) water and a boiling acetone/water mixture [3:4 (v/v)], respectively. *cis*-[Dichlorobis(hydroxylamine)platinum(II)] [**3** (*cis*)] was prepared from Li$_2$[PtCl$_4$] and NH$_2$OH·HCl in the presence of lithium acetate in water (Stetsenko et al., 1989), whereas *trans*-[dichlorobis(hydroxylamine)platinum(II)] [**4** (*trans*)] was prepared by heating of [Pt(NH$_2$OH)$_4$](OH)$_2$ in 0.1 M HCl (Uhlenhut, 1900); both complexes were recrystallized from 0.1 M HCl.

Cell Lines and Culture Conditions. Human CH1 (ovarian carcinoma) and SW480 (colon carcinoma) cells were kindly provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK) and Brigitte Marian (Institute of Cancer Research, Medical University of Vienna, Austria), respectively. Cells were grown in 75-cm^2 culture flasks (Iwaki/Asahi Technoglass, Gyouda, Japan) as adherent monolayer cultures in complete culture medium [i.e., minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% nonessential amino acids (100×) (all purchased from Invitrogen, Paisley, UK)]. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$.
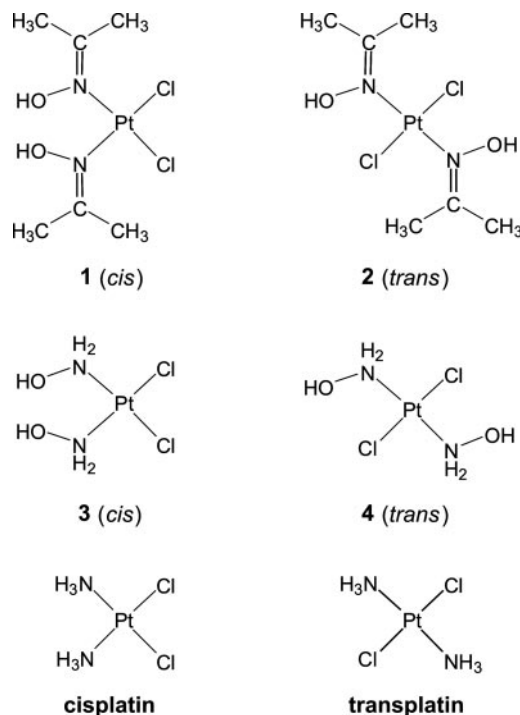

Fig. 1. Structures of acetoxime platinum complexes **1** (*cis*) and **2** (*trans*), hydroxylamine platinum complexes **3** (*cis*) and **4** (*trans*), and cisplatin and transplatin.

Cytotoxicity in Cancer Cell Lines. Cytotoxicity was determined by means of a colorimetric microculture assay (MTT assay). CH1 and SW480 cells were harvested from culture flasks by trypsinization and seeded into 96-well microculture plates (Iwaki/Asahi Technoglass, Gyouda, Japan) in cell densities of 2.5×10^3 and 3×10^3 cells/well, respectively, to ensure exponential growth throughout drug exposure. After a 24-h preincubation, cells were exposed to serial dilutions of the test compounds in 200 μl/well complete culture medium for 96 h. At the end of exposure, drug solutions were replaced by 150 μl/well RPMI 1640 culture medium (supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine) plus 20 μl/well MTT solution in phosphate-buffered saline (5 mg/ml). After incubation for 4 h, the medium/MTT mixtures were removed, and the formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in 150 μl of dimethyl sulfoxide per well. Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic). The quantity of vital cells was expressed in terms of T/C values by comparison with untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising at least six microcultures per concentration level.

Chemicals, Electrolytes, and Samples for Capillary Zone Electrophoresis. Sodium hydroxide, sodium dihydrogen phosphate, dGMP, and HEPES were of analytical grade and were obtained from Fluka (Buchs, Switzerland). Disodium hydrogen phosphate was purchased from Riedel-de Haen (Seelze, Germany). High purity water used throughout this work was obtained from a Millipore Synergy 185 UV Ultrapure Water system (Molsheim, France).
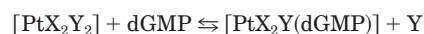
For incubation, a 20 mM HEPES buffer at physiological pH (7.4) and 37°C was chosen. Because HEPES absorbs in the UV range, a different buffer had to be used as background electrolyte (BGE) for the electrophoretic separations—a 20 mM phosphate buffer, pH 7.4, was used for this purpose. The incubation buffer and BGE were passed through a 0.45-μm disposable membrane filter (Sartorius, Goettingen, Germany) before being injected hydrodynamically into the capillary zone electrophoresis system.

The platinum complexes were dissolved in the dGMP-containing incubation buffer, constituting a drug-to-dGMP ratio of 1:2. Due to poor solubility, an initial concentration of 0.2 mM was chosen for **2** (*trans*) compared with 0.5 mM for the other compounds.

Studies on dGMP Binding by Capillary Zone Electrophoresis. Capillary zone electrophoresis experiments were performed on an HP^{3D} capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with an on-column diode-array detector. For all measurements, uncoated fused silica capillaries of 50 cm total length (50 μm i.d., 42-cm effective length) were used (Polymicro Technologies, Phoenix, AZ). Capillary and sample tray were thermostated at 37°C, injections were performed by applying a pressure of 10 mbar for 15 s, and a constant voltage of 15 kV was used for all separations (the resulting current was approximately 25 μA). Detection was carried out at 200 and 254 nm. Before first use, the capillary was flushed with 0.1 M HCl, water, 1 M NaOH, and again with water for 10 min each and then equilibrated with the BGE for 10 min. Before each injection, the capillary was purged with 0.1 M NaOH and water for 2 min each and finally conditioned with the BGE for 3 min.

The rate of binding to dGMP was measured by monitoring the decrease of the peak area response corresponding to the dGMP signal. The peak areas were normalized using the area of the incubation buffer signal as an internal standard. The kinetic series was repeated at least four times for each of the compounds.

To find an equation that most closely describes the behavior and character of kinetic curves and fits the experimental data, regression analysis was undertaken [natural logarithm of the dGMP concentration (i.e., its peak area) versus time]. From a schematic standpoint, the first stage of reaction can be expressed as follows:

$$[PtX_2Y_2] + dGMP \leftrightarrows [PtX_2Y(dGMP)] + Y$$

or

$$A + B \leftrightarrows C + D \tag{1}$$

Second stage of the reaction:

$$[PtX_2Y(dGMP)] + dGMP \leftrightarrows [PtX_2(dGMP)_2] + Y$$

or

$$C + B \leftrightarrows D + E \tag{2}$$

The rate of the chemical reaction is determined by the slowest stage of the whole process. For bimolecular reactions, as written in eq. 1, the rate of the reaction can be expressed as

$$-\frac{d[B]}{d[t]} = k_1[A][B] \tag{3}$$

for its first stage, and for the second stage (assuming that $[C] > [B]$, and pseudo–first-order consequently):

$$-\frac{d[B]}{d[t]} = k_2'[B] \tag{4}$$

where k_1 is the rate constant of the first stage, and k_2' is a pseudo rate constant of the second stage of the reaction.

Preliminary estimations of the rate constants for the both stages have discovered that the rate constant of the first stage is much higher than the rate constant of the second stage. This means that the rate constant of the second stage determines the rate of the complete reaction, and in the following speculations we define k_2' as the pseudo rate constant for the whole process (k_{bind}). Pseudo rate constants were calculated from fitted curves; half-lives were determined graphically.

Starting Materials for DNA Interaction Studies. For all examinations, a stock solution of the investigated compounds was prepared in double-distilled water and stored immediately at $-20°C$. Plasmid pTZ18u (2860 bp) was from Bio-Rad Laboratories (Munich, Germany). Plasmid P5 (3016 bp) was a gift from Dr. M. Ried (CRE-LUX GmbH, Munich/Martinsried, Germany). The plasmids were transformed in XL1 blue cells, isolated and purified according to standard procedures and dissolved in TE buffer. Restriction endonuclease PvuII and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs (Ipswich, MA). Restriction endonuclease EcoRI and molecular weight marker GeneRuler 50-bp DNA Ladder were from Fermentas (Burlington, ON, Canada). All radioactive products were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Changes in DNA Secondary Structure and DNA Modification Degree. Plasmid P5 was cleaved with EcoRI and PvuII to generate a linear double-stranded 177-bp fragment. The fragment was eluted from an agarose gel after electrophoretic separation and 3′-end-labeled by the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP.

For each time point of the kinetics analysis, 1 μg of plasmid pTZ18u and 1.6 fmol of radioactively end-labeled 177-bp fragment and either one of the compounds at a final concentration of 60 μM were incubated separately in 40 μl of 0.1 × TE buffer (1 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA) at 37°C. For detection of changes in DNA secondary structure, 5 μl of 5×"blue juice" sample buffer (final, 2.5% glycerol, 0.5% SDS, 10 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol) were added to a 20-μl aliquot of a specific time point. The reaction products were separated immediately in a 1% agarose gel in TBE buffer at 3 V/cm. The gel was stained with 0.2 μg/ml EtBr in 1 × TBE, illuminated by UV light and photographed using a gel documentation system from Vilber Lourmat (Torcy Z.I. Sud, Marne-La-Vallee, France). To visualize the DNA modification degree, 10-μl aliquots of each time point were mixed with 2.5 μl of

5×"blue-juice" sample buffer. The samples were analyzed in a 4% polyacrylamide gel in 1× TBE buffer, 0.1% SDS at 15 V/cm. After electrophoresis, the gel was fixed in 7% acetic acid, 4% glycerol for 20 min and dried for 2 h at 65°C under vacuum. The gel was exposed to an X-ray film overnight at −70°C. Analyses of DNA secondary structure and of DNA modification degree were performed at least three times with virtually identical results.

Interstrand Cross-Link Assay. To analyze the ability of examined complexes to form ICLs, 0.8 fmol of radioactively end-labeled 177-bp fragment (see above) were incubated in $0.1 \times$ TE buffer as described above at a final concentration of 60 μM and a final volume of 20 μl per sample. After incubation, all samples were instantly evaporated to complete dryness in a SpeedVac (Thermo Electron, Waltham, MA) and resuspended in 10 μl of loading dye (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromphenol blue), heated for 3 min at 95°C and chilled in ice. The reaction products were separated in a denaturing 4% polyacrylamide gel, 7 M urea, $1 \times$ TBE, at 10 V/cm for 1 h. After fixing the gel in 7% acetic acid and 4% glycerol for 20 min and drying for 2 h at 65°C under vacuum, it was exposed to an X-ray film at −70°C for an appropriate time. ICL assays were repeated at least two times.

TABLE 1
Cytotoxicity of acetoxime platinum complexes **1** (*cis*) and **2** (*trans*) and hydroxylamine platinum complexes **3** (*cis*) and **4** (*trans*) compared with cisplatin and transplatin in two human cancer cell lines

Presented are the 50% inhibitory concentrations in CH1 and SW480 cells in the MTT assay. Values are the means ± S.D. obtained from at least three independent experiments.

Compound	IC$_{50}$	
	CH1	SW480
	μM	
1 (*cis*)	2.7 ± 0.7	3.4 ± 0.3
2 (*trans*)	0.17 ± 0.09	0.22 ± 0.05
3 (*cis*)	0.68 ± 0.23	12 ± 1
4 (*trans*)	51 ± 15	90 ± 3
Cisplatin	0.14 ± 0.03	3.3 ± 0.4
Transplatin	15 ± 2	19 ± 3

Results

Cytotoxicity. The cytotoxic potencies of the acetoxime and hydroxylamine platinum complexes were compared with those of cisplatin and transplatin in the cisplatin-sensitive ovarian carcinoma cell line CH1 and the inherently cisplatin-resistant colon carcinoma cell line SW480 by means of the colorimetric MTT assay. IC$_{50}$ values are listed in Table 1, and complete concentration-effect curves are depicted in Fig. 2.

In accordance with ample evidence from the literature, transplatin is much less cytotoxic than cisplatin, although the difference between their potencies is much less pronounced in SW480 cells than in CH1 cells, which differ tremendously in their cisplatin sensitivity. In sharp contrast, the acetoxime complex **2** (*trans*) is roughly 15 times more potent than the corresponding geometric isomer **1** (*cis*) in both cell lines. In CH1 cells, the cytotoxicity of **2** (*trans*) is comparable with that of cisplatin, but it is even more potent than cisplatin in SW480 cells by an order of magnitude, indicating that the mechanisms causing the inherent cisplatin resistance of the latter cell line do not affect the activity of **2** (*trans*).

In the case of the hydroxylamine complexes, **3** (*cis*) is superior to **4** (*trans*), concordant with the classic structure-activity relationships derived from the cisplatin/transplatin couple. The differences between the cytotoxic potencies of **3** (*cis*) and **4** (*trans*) are similar to those between cisplatin and transplatin, their IC$_{50}$ values being shifted to higher concentrations, however. In contrast to acetoxime complex **1** (*cis*), hydroxylamine complex **3** (*cis*) closely parallels cisplatin insofar as a certain fraction of SW480 cells (up to 10%) resists rather high concentrations (3–12 times the respective IC$_{50}$), resulting in a characteristic shoulder in the concentration-effect curves (Fig. 2), suggesting an as-yet-unidentified resistance mechanism for both compounds.
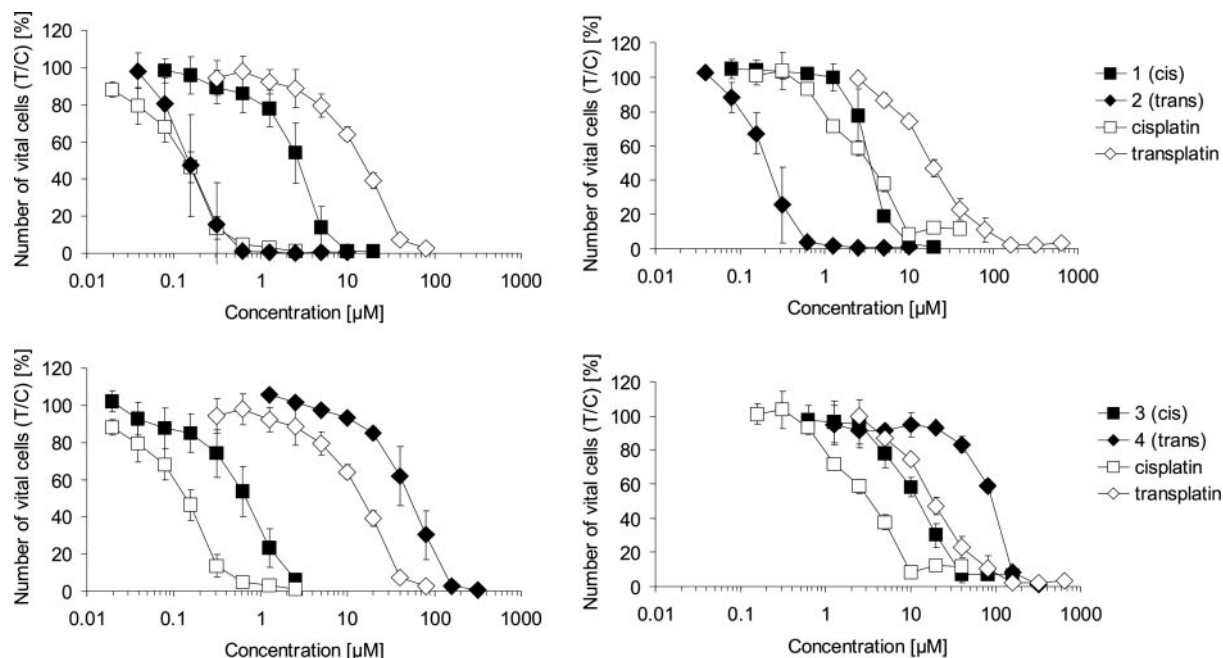

Fig. 2. Concentration-effect curves of acetoxime platinum complexes **1** (*cis*) and **2** (*trans*) (top) and hydroxylamine platinum complexes **3** (*cis*) and **4** (*trans*) (bottom) compared with cisplatin and transplatin in the human cancer cell lines CH1 (left) and SW480 (right). Values were obtained by the MTT assay and are the means ± S.D. from at least three independent experiments.

Binding Behavior toward dGMP. Capillary electrophoresis has often been applied to the analysis of platinum group complexes as well as their interaction with biomolecules in recent years (Hartinger et al., 2003; Timerbaev et al., 2006). DNA is considered the critical target for platinum complexes, and competitive studies including all four nucleobases confirmed guanine (and to a lesser extent adenine) as the preferred binding partner for the metal complexes—adduct formation takes place mainly via the *N7* of the nucleobase (Martin, 1999). Therefore, it was reasonable to compare the binding behavior of the complexes included in this study toward the model compound dGMP.

Reactivity decreases in the following order (based on the pseudo rate constants and the half-life of the dGMP peak): **3** (*cis*) > **4** (*trans*) > cisplatin > transplatin > **2** (*trans*) > **1** (*cis*) (Table 2). When comparing the binding kinetics of cisplatin and transplatin, it can be seen that transplatin reacts slightly faster than its *cis* analog during the first hours of incubation. Nevertheless, the reaction speed decreases as the amount of *trans*-bound dGMP increases, indicating that the attachment of a second dGMP to the *cis* isomer is kinetically favored (Fig. 3). This might be due to faster exchange of the second chloro ligand in cisplatin with water, because aquation is considered a prerequisite for adduct formation (Martin, 1999; Zenker et al., 2000).

On the contrary, the acetoxime-containing complexes **1** (*cis*) and **2** (*trans*) show a different behavior: The *trans* isomer binds faster to dGMP than the *cis* form, not only in the beginning but throughout the whole period of incubation. However, because of higher hydrolytic stability, binding

progresses at a much slower rate compared with the other compounds (Table 2). In the case of hydroxylamine-containing complexes **3** (*cis*) and **4** (*trans*), a similar observation as for cisplatin and transplatin can be made: Binding of 1 Eq of dGMP progresses slightly faster for the *trans* compound, whereas the *cis* form shows stronger interaction toward a second dGMP, as reflected by the pseudo rate constant of the overall process.

In general, because of the complex nature of the reactions taking place in the sample simultaneously (aquation, oligomerization, dGMP binding), not all minor peaks in the electropherograms could be assigned to an exact structure. In any case, detection at 254 nm and analysis of the spectral patterns and migration times of the major peaks enabled us to clearly distinguish dGMP adducts (Fig. 4) and therefore also determine pseudo rate constants and half-lives (Table 2).

Alterations of DNA Secondary Structure and Reactivity with DNA. To examine the alterations of DNA secondary structure for the investigated compounds, kinetic studies of either one of the complexes with plasmid DNA were performed.

It is largely documented that platinum-based complexes can untwist, locally melt, and/or bend dsDNA, depending on the kind of the specific adducts formed on DNA (Lepre and Lippard, 1990); for example, monofunctional or intercalating adducts may untwist dsDNA, whereas bifunctional adducts (intra- as well as interstrand cross-links), in addition, bend DNA. Conversely, analyzing the DNA secondary structure may provide valuable clues about the kind of the DNA adducts. Changes of DNA secondary structures can easily be monitored by evaluating the electrophoretic migration pattern of a circular dsDNA plasmid in neutral agarose gels. Adducts that untwist dsDNA effect a slower migration of the negatively "supercoiled form" (sc) of the plasmid as a result of partial relief of the torsional stress and consequent relaxing of the compact sc form; a faster migration of the nicked, "open circular" form (oc) of a plasmid, on the other hand, is consistent with adducts that compact or apparently "condense" dsDNA (Cohen et al., 1979).

Hence, to examine the time-dependent alterations of DNA secondary structure for the investigated compounds, kinetic studies of the complexes with plasmid DNA were performed.

TABLE 2
Summary of pseudo rate constants k_{bind} (with correlation coefficent R) and graphically assessed half-lives ($\tau_{1/2}$) for the dGMP binding of the studied platinum compounds

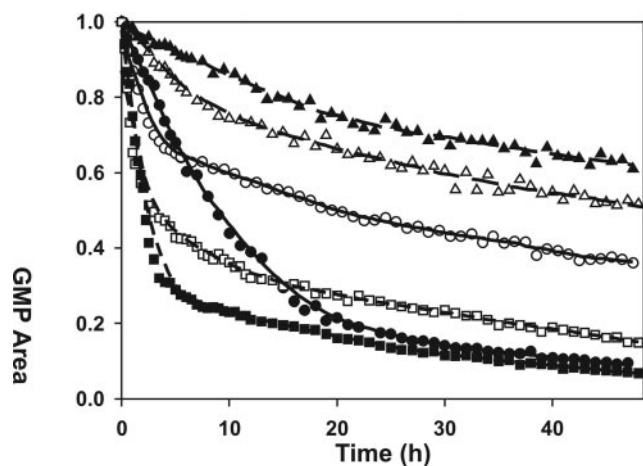| Compound	k_{bind}	R	$\tau_{1/2}$
	mol/h		*h*
1 (*cis*)	0.0040	0.9995	>72
2 (*trans*)	0.0088	0.9989	50.1 ± 1.9
3 (*cis*)	0.3392	0.9971	2.4 ± 0.2
4 (*trans*)	0.2696	0.9909	3.4 ± 0.3
Cisplatin	0.0602	0.9983	8.9 ± 0.7
Transplatin	0.0116	0.9998	20.0 ± 0.9

Fig. 3. Time courses of the dGMP binding reaction of all studied substances. The solid lines correspond to cisplatin (●) and transplatin (○), the long dashes to acetoxime complexes **1** (*cis*) (▲) and **2** (*trans*) (△), and the short dashes to hydroxylamine complexes **3** (*cis*) (■) and **4** (*trans*) (□).
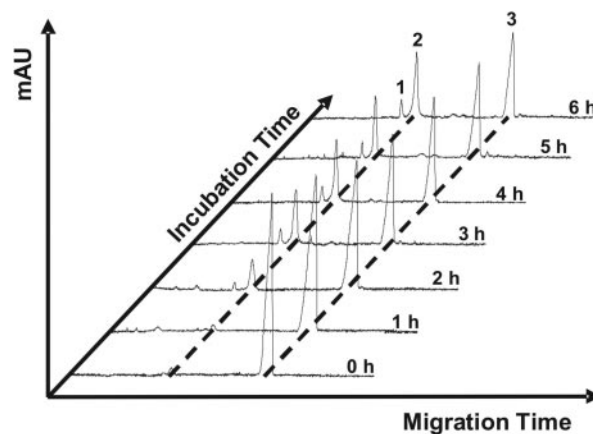

Fig. 4. Monitoring of the dGMP-binding reaction of compound **3** (*cis*). Separation voltage, 15 kV; detection wavelength, 254 nm. Other conditions: see *Materials and Methods*. Peak identification: 1, monoadduct; 2, bisadduct; 3, dGMP.

However, because different DNA-interacting drugs may induce secondary structure changes of different magnitudes, monitoring the kinetics of secondary structures does not necessarily reflect the degree of DNA modification or reactivity of the drug. For this reason, to directly visualize the extent of DNA modification, we performed an additional direct control of reactivity. A linear, radioactively end-labeled 177-bp dsDNA fragment was included in each reaction, and its migration was analyzed in a neutral polyacrylamide gel. The modification of the linear dsDNA fragment leads to increased molecular weight and to additional positive charges on DNA, resulting in upward shifting of the fragment in the gel analysis, which reflects the modification degree of all DNA in the reaction. Consequently, with this set-up, it is possible to visualize both induced alterations of DNA structure *and* the reactivity of complexes at the same time.

Figure 5 shows the electrophoretic pattern of plasmid DNA incubated with 60 μM compound **1** (*cis*) or **2** (*trans*) at 1-h time intervals for up to 7 h. Both compounds effect relaxation of the sc form and mobilization of the oc form of the plasmid. These differences in plasmid migration are consistent with induced untwisting and apparent DNA condensation, respectively. The DNA condensation may be caused by bending [i.e., rigid, directed but offset bends or flexible hinge joints (see *Discussion*)].

Compound **2** (*trans*) (Fig. 5C) is evidently more inefficient than compound **1** (*cis*) (Fig. 5A) in inducing changes in DNA secondary structure. This may be due either to slower kinetics or to a weaker extent of structural changes at individual adducts (or both). However, the moderate shifting of the radioactively end-labeled dsDNA fragment in gel analysis shown in Fig. 5D compared with compound **1** (*cis*) (Fig. 5B), reflecting a smaller DNA modification degree, reveals that

the inefficacy of inducing changes in DNA secondary structure may be rather due to slower kinetics. Hence, the two isomers are likely to form adducts of comparable average impact on the DNA structure, albeit with different speed.

Besides the general ability of compound **1** (*cis*) to react with and induce changes in secondary structures of DNA faster than compound **2** (*trans*), an additional difference became visible. By comparing the mobilization of the oc forms of the plasmid at nearly equivalent global untwisting degrees (Fig. 5C, lane 8, versus Fig. 5A, lane 5), it is obvious that bending (as defined above) induced by compound **2** (*trans*) is less pronounced. This might indicate that, if this bending were due to closure to bifunctional adducts of compound **2** (*trans*), this reaction would also be kinetically impaired compared with compound **1** (*cis*).

The results of the interaction of plasmid DNA and a radioactively end-labeled dsDNA fragment with complexes of the hydroxylamine type, **3** (*cis*) and **4** (*trans*), are shown in Fig. 6. Both complexes displayed a much higher ability to induce changes in DNA secondary structure than the acetoxime compounds. Beyond 1 or 2 h of incubation, respectively, compounds **3** (*cis*) and **4** (*trans*) untwisted the plasmid to positive supercoils. The marked formation of adducts with plasmid DNA was accompanied by a distinctive shifting of the included DNA fragment for both compounds shown in Fig. 6, B and D. In addition, the **4** (*trans*) isomer showed a different migration behavior of the open circular form of the plasmid, analogous to the **2** (*trans*) isomer of the acetoxime type.

As a control, plasmid DNA was also incubated with cisplatin and transplatin. As expected, both compounds effected relaxation of the sc form and mobilization of the oc form of the plasmid, whereas transplatin showed a slightly higher efficiency to induce secondary structures as contrasted to
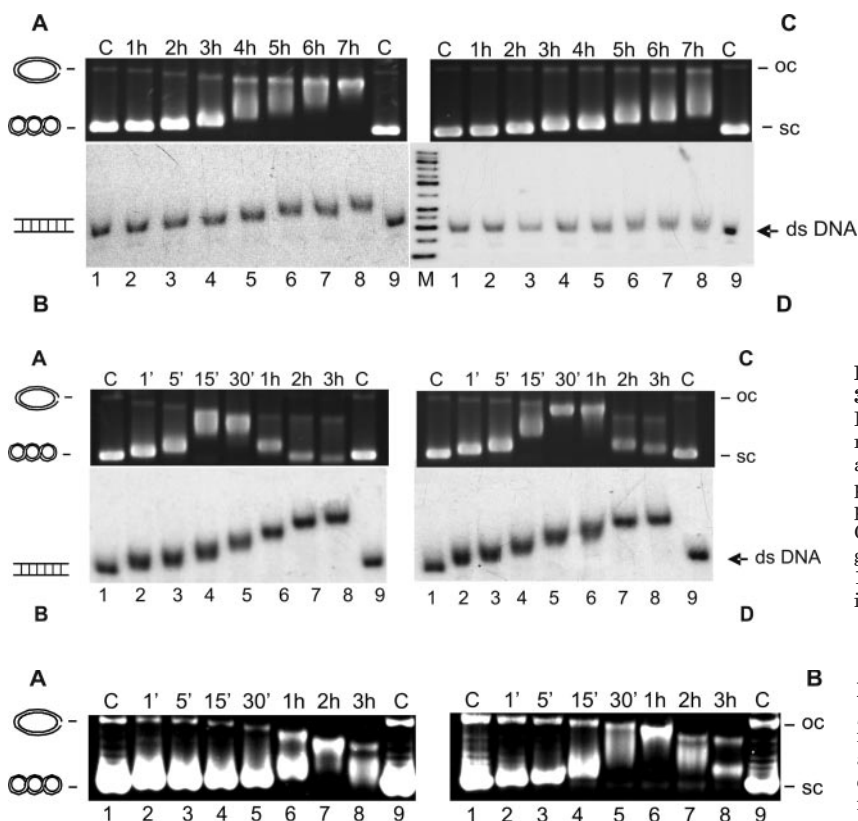

Fig. 5. Interaction of acetoxime platinum complexes **1** (*cis*) (A and B) and **2** (*trans*) (C and D) with dsDNA. Products of plasmid pTZ18u (A and C) and of a linear, radioactively labeled 177-bp DNA fragment (B and D) after incubation with 60 μM compound **1** (*cis*) and compound **2** (*trans*), respectively, for the indicated time points (lanes 2–8) and electrophoretic separation. A and C, 1% agarose gel stained with EtBr; B and D, autoradiograph of a dried, neutral 4% polyacrylamide gel. C at lane 1, control DNA, not incubated; C at lane 9, control DNA, incubated for 7 h.

Fig. 6. Interaction of hydroxylamine platinum complexes **3** (*cis*) (A and B) and **4** (*trans*) (C and D) with dsDNA. Products of plasmid pTZ18u (A and C) and of a linear, radioactively labeled 177 bp DNA fragment (B and D) after incubation with 60 μM compound **3** (*cis*) and compound, **4** (*trans*), respectively, for the indicated time points (lanes 2–8) and electrophoretic separation. A and C, 1% agarose gel stained with EtBr; B and D, autoradiograph of a dried, neutral 4% polyacrylamide gel. C at lane 1, control DNA, not incubated; C at lane 9, control DNA, incubated for 3 h.
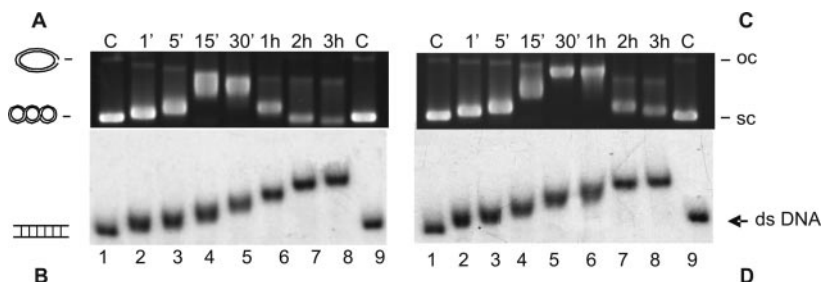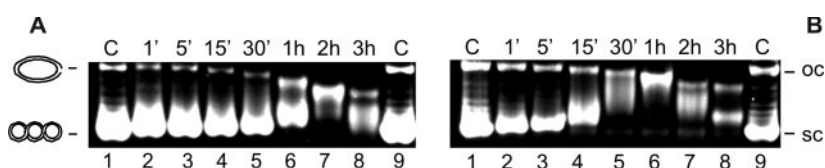
Fig. 7. Products of plasmid DNA after incubation with 60 μM cisplatin (A) and transplatin (B), respectively, for the indicated time points (lanes 2–8) and electrophoretic separation in 1% agarose gel stained with EtBr. C at lane 1, control DNA, not incubated; C at lane 9, control DNA, incubated for 3 h

cisplatin (Fig. 7). This parallels the known higher reactivity of transplatin against DNA (Farrell et al., 1992). In accordance to the *trans* isomers **2** (*trans*) and **4** (*trans*), it is apparent that, at corresponding untwisting extent, the mobilization of the oc form of the plasmid caused by transplatin was less pronounced than that caused by cisplatin.

With both substrates, dGMP and DNA fragment, the results show coincidence regarding the comparative reactivities between each pair; i.e., **3** (*cis*)/**4** (*trans*) prevail over cisplatin/transplatin, which beat **1** (*cis*)/**2** (*trans*). Likewise, the reactivity order within the pair **3** (*cis*)/**4** (*trans*) is matching between dGMP and DNA. However, the reactivity order within the other two pairs is reversed depending on the substrate. Besides possible, inevitable imprecision of the results with the DNA fragment, we suggest that the DNA results reflect rather faithfully the reactivity of the compounds. Investigations of reactivity with dGMP may not mirror the rate of formation of DNA adducts in a representative way, because the rate of formation of dGMP adducts is governed by the complete translational and rotational freedom of the soluble substrate dGMP in contrast with the sterically constrained and spatially well defined target bases in DNA.

Formation of Interstrand Cross-Links. Intrastrand as well as interstrand cross-links are known to bend DNA. To examine whether the investigated compounds can form ICLs, a radioactively 3′-end-labeled 177-bp DNA fragment was incubated with either one of the investigated compounds, and the reaction products were analyzed in a denaturing urea-polyacrylamide gel. On the basis of this set up, the former double-stranded DNA molecule appears single-stranded at lower regions of the gel when no ICLs are being formed. If the investigated complex is able to form ICLs, a new distinct band with lower mobility is visible in the gel, representing a former double-stranded DNA molecule with a minimum of one interstrand cross-link that is not able to be separated in a denaturing polyacryamide gel.

Figure 8 shows the results of the reaction of the hydroxyl-amine platinum complexes **3** (*cis*) and **4** (*trans*) with linear dsDNA after separation in a denaturing urea-polyacrylamide gel. Both complexes showed a clear increase of ICL formation over time. Beyond 15 min for compound **3** (*cis*) or 1 h for compound **4** (*trans*), all DNA molecules contained at least one

Fig. 8. Formation of ICLs by hydroxylamine platinum complexes **3** (*cis*) and **4** (*trans*). Products of a linear, radioactively labeled 177-bp DNA fragment after incubation with 60 μM compound **3** (*cis*) (A) and compound **4** (*trans*) (B), respectively, for the indicated time points (lanes 2–8) and electrophoretic separation in a denaturing 4% polyacrylamide gel; depicted are the autoradiographs of the dried gels. C at lane 1, control DNA, not incubated; C at lane 9, control DNA, incubated for 3 h.

ICL, displayed in discrete upward shifts of DNA. In general, further incubation led to faster migration of the ICL-connected DNA strands. This might be due to an increasing compactness of the DNA strands containing more ICLs, therefore mimicking the form and migration properties of linear dsDNA.

As contrasted with the clear formation of interstrand cross-links induced by compounds **3** (*cis*) and **4** (*trans*), the acetoxime platinum complexes **1** (*cis*) and **2** (*trans*) showed no formation of ICLs whatsoever for the time points investigated (1–7 h) (data not shown).

Discussion

We have discovered that **2** (*trans*) is a new unconventional platinum compound violating the classic structure-activity relationship, insofar as its cytotoxicity is comparable with cisplatin in the ovarian carcinoma cell line CH1 and even superior to cisplatin by 1 order of magnitude in the rather cisplatin-insensitive colon carcinoma cell line SW480, indicating a potential of overcoming primary cisplatin resistance. This again emphasizes the great relevance of active *trans* complexes for the development of new platinum-based anti-cancer drugs, because non–cross-resistance to cisplatin is common to most of these compounds. The observation that **2** (*trans*) is 15 to 16 times more cytotoxic than **1** (*cis*) is striking, because the *cis* counterparts of active *trans* complexes investigated by other authors are not substantially less active (e.g., Farrell et al., 1992; Farrell, 1996), indicating that the substitution of ammine by appropriate ligands usually activates the *trans* geometry without severely impairing the activity of the *cis* congener. Hence, to our knowledge, this is the first successful *reversion* of the structure-activity relationship of the *cis* and *trans* geometry.

A synopsis with the active *trans*-platinum complexes reported by other authors (see Introduction) reveals that **2** (*trans*) shares with both iminoether and acetimine complexes the azomethine moiety C=N and the planarity around the nitrogen donor atom resulting from its sp² hybridization. This also applies to *N*-heterocyclic complexes, but the involvement of the nitrogen donor atom in an aromatic ring system strongly distinguishes them from the former. Furthermore, the acetoxime ligand shares with the branched aliphatic amine and the acetimine type of ligands the ramification of the alkyl residue at the proximate carbon atom. The minimal structural requirement for an active *trans*-platinum complex, as inferred from this synopsis, is the presence of at least one of the following characteristics: 1) an sp²-hybridized nitrogen donor atom, 2) a branched aliphatic chain attached to the nitrogen donor atom, or 3) a nitrogen donor atom integrated into a cycloaliphatic amine.

Although the acetoxime complex **2** (*trans*) resembles the acetimine complexes of Boccarelli et al. (2006) in two crucial respects (i.e., the planar azomethine and the branched aliphatic moiety), it differs from them (and from all other examples of active *trans*-platinum complexes) by the formal substitution of the nitrogen-bound hydrogen by hydroxyl groups. Because this renders the compound a stronger H-bonding donor than conventional amine complexes, an involvement of hydrogen bonding in the DNA interactions (e.g., in stabilizing monofunctional adducts) should be considered. Moreover, the OH acidity of the metal-bound acetoximes is
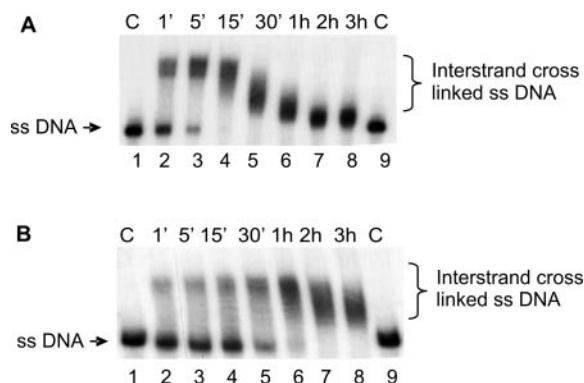
significant (pK_{a1}, 6–7) (Kukushkin et al., 1996), but although this acidity constitutes a major difference from other active *trans*-platinum complexes, the sole presence of a hydroxyl group bound to the nitrogen donor atom is neither sufficient nor essential for activity of the *trans* isomer, as can be inferred from the classic structure-activity relationship of the [PtCl$_2$(hydroxylamine)$_2$] couple, **3** (*cis*) and **4** (*trans*).

The reactivity of the compounds has been investigated by monitoring the reaction with dGMP and with a DNA fragment. Although **2** (*trans*) was the least reactive with DNA, it was the most cytotoxic of the investigated compounds. The fact that slowly reacting compounds display a rather strong cytotoxicity or, inversely, that strongly reacting compounds may be devoid of biologic activity is striking but not new or astonishing. For instance, it has been shown repeatedly that transplatin reacts approximately 2.5-fold more efficiently than cisplatin with both calf thymus and plasmid DNA (e.g., see Farrell et al., 1992), yet without favorable impact on its cytotoxicity. Likewise, the cytotoxity of *trans*-dichlorobis(*E*-iminoether)platinum(II), *trans*-EE, which is closely related to **2** (*trans*), is comparable with cisplatin in the P388 leukemia system (Coluccia et al., 1995), although cisplatin displays significantly faster reaction kinetics with calf thymus DNA than *trans*-EE (Coluccia et al., 1995; Žaludová et al., 1997). It may be that efficient reaction of a compound even with the *cellular* DNA cannot bring about increased cytotoxicity if its adducts are rapidly removed by repair systems. Instead, formation of repair-resistant adducts that actively lead to programmed cell death is critical for the activity of the compounds (Zorbas and Keppler, 2005). Hence, the cytotoxicity of active *trans* isomers in general, and the isomer **2** (*trans*) investigated in this study in particular, may therefore rely on the formation of particularly potent adducts.

In fact, the cytotoxic power of platinum complexes has been associated with adducts that induce particular secondary structures of DNA (Eastman, 1999; Kartalou and Essigmann, 2001). We found that our novel compounds were able to induce changes of DNA structure (i.e., visible DNA relaxation and DNA condensation). Relaxation of the sc DNA was obviously brought about by *local untwisting* at the sites of adducts. In accord to numerous investigations of platinum compounds, the detected untwisting is consistent with formation of monofunctional adducts at purine nucleobases. Hence, the compounds of both types (hydroxylamine and acetoxime) may form monofunctional adducts. Condensed circular DNA modified with platinum complexes, first described for cisplatin and transplatin by Cohen et al. (1979), was recognized as being caused by multiple rigid or flexible *bends* not in phase with the DNA periodicity, leading to apparent diminished diameter of circular DNA (Bellon and Lippard, 1990; Bellon et al., 1991). We cannot distinguish between the two variants of bending, stable or flexible, in this study. Bending may be caused by bifunctional adducts, either intrastrand (Takahara et al., 1995) or interstrand (Huang et al., 1995) cross-links. In addition, some compounds with the *trans* geometry have been reported to effect bending by monofunctional coordination (Zakovska et al., 1998; Kasparkova et al., 2003; Novakova et al., 2003). Nevertheless, at least for the *trans* compounds with heterocyclic ligands, (stacking) interactions of these ligands with DNA were also discussed, which might give rise to "pseudobifunctional" adducts (Zakovska et al., 1998). Hence, compounds of

the hydroxylamine type might have caused bending by any kind of adducts. On the other hand, because the acetoxime compounds lacked the capacity to form ICLs, bending with these compounds cannot have been effected by this type of cross-links, but rather by bifunctional *intrastrand* cross-links or, in the case of **2** (*trans*), even *monofunctional* adducts.

We observed that the induced condensation of the oc form was less pronounced with all investigated *trans* isomers than with all investigated *cis* isomers at comparable global untwisting, best visible with the pair **1** (*cis*)/**2** (*trans*) (Fig. 5). If *bifunctional* adducts were the cause of bending, this might be an indication of a slower reaction of the second platinum valence of the *trans* isomers compared with the *cis* isomers. Interestingly though, the same observation has been made with *trans*- versus *cis*-EE (see Fig. 5 in Žaludová et al., 1997) [i.e., with a structurally similar, also active *trans* compound that is known to cause bending by abundant *monofunctional* adducts (Novakova et al., 2003)]. By analogy, therefore, it is tempting to speculate that, in our case, bending might have been effected by monofunctional adducts as well. This, however, will be the subject of future studies.

Which structural feature might constitute the high cytotoxicity of our active compounds? Intrastrand cross-links [such as the 1,2-d(GpG) adduct of cisplatin, which displays unique structural features, the main characteristic of which is a rigid, directed bend of 30° to 35° into the major groove of dsDNA (Jamieson and Lippard, 1999)] may be important cytotoxic lesions. On the other hand, ICLs, although minor adducts in general (i.e., ~2% of all cisplatin adducts), have never been excluded as possible lethal lesions of platinum complexes. In fact, ICLs, like intrastrand adducts, may be equally important cytotoxic adducts under certain conditions (Zdraveski et al., 2000; Aloyz et al., 2002). **3** (*cis*) and **4** (*trans*) readily form ICLs. Because **3** (*cis*) displays a fairly high cytotoxicity, we cannot exclude that, in this case, ICLs may have contributed to the biologic effect. However, ICLs are certainly *not sufficient* for cytotoxicity, because **4** (*trans*), which shows ICL formation kinetics comparable with those of **3** (*cis*), was quite inactive. In contrast, we could detect no ICLs for the time period investigated with the acetoxime compounds, particularly with the active **2** (*trans*) compound. Therefore, ICLs seem definitely to be *not necessary* for the superb cytotoxic activity of the **2** (*trans*). It is noteworthy that the highly cytotoxic and antitumoral *trans*-EE was also found to have a very small DNA interstrand cross-linking efficacy (Coluccia et al., 1995; Žaludová et al., 1997).

If monofunctional adducts constitute a major fraction of the **2** (*trans*) lesions, they may contribute significantly to cytotoxicity as well. As was shown for monofunctional adducts of *trans*-EE (Novakova et al., 2003), proteins like histone H1 may be readily captured by the available valence of the platinum giving rise to ternary DNA-drug-protein complexes. Such complexes inhibit in vitro DNA polymerization and, most importantly, removal of adducts by the nucleotide excision-repair system. Resultant prolonged persistence of such adducts may facilitate in vivo the onset of cell death mechanisms. Future investigations will evaluate this possibility for our compounds.

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