

Molecular Aspects of Antitumor Effects of a New Platinum(IV) Drug

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ABSTRACT

The new platinum(IV) complex *cis,trans,cis*-[PtCl₂(CH₃COO)₂-(NH₃)(1-adamantylamine)] [adamplatin(IV)] seems promising for the perspective application in therapy of corresponding tumors. It is therefore of great interest to understand details of mechanisms underlying its biological efficacy. Cellular uptake of the drug, alterations in the target DNA induced by platinum drugs along with processing of platinum-induced damage to DNA and drug inactivation by sulfur-containing compounds belong to major pharmacological factors affecting antitumor effects of platinum compounds. We examined in the present work the significance of these factors in the mechanism of antitumor effects of adamplatin(IV) and compared the results with those of the parallel studies performed with “conventional” cisplatin. The results show that deactivation of adamplatin(IV) by sulfur-containing compounds (such as glutathione or metallothio-

neins) is likely to play a less significant role in the mechanism of resistance of tumor cells to adamplatin(IV) in contrast to the role of these reactions in the effects of cisplatin. Moreover, the treatment of tumor cells with adamplatin(IV) does not result in DNA modifications that would be markedly different from those produced by cisplatin. In contrast, the effects of other factors, such as enhanced accumulation of the drug in cells, strong inhibition of DNA polymerization by these adducts, lowered DNA repair, and DNA-protein cross-linking are different from the effects of these factors in the mechanism underlying activity of cisplatin. Hence, the differences between effects of adamplatin(IV) and cisplatin observed in the present work on molecular level may help understand the unique activity of adamplatin(IV).

Platinum antitumor compounds, such as cisplatin [*cis*-diamminedichloroplatinum(II)] (Fig. 1A) and its analogs, are widely used in the treatment of testicular and ovarian cancers and a variety of other human solid tumors. Much progress had been made to understand the mechanisms involved in antitumor effects of platinum compounds and drug resistance, which are multifactorial processes (Brabec, 2002;

Fuertes et al., 2003). The target for platinum antitumor compounds is DNA, to which they bind efficiently, forming a variety of adducts that block replication and transcription and induce cell death (Johnson et al., 1989). The nature of DNA adducts affects a number of transduction pathways and triggers apoptosis or necrosis in tumor cells (Fuertes et al., 2003). In addition to alterations in the target and processing of platinum-induced damage to DNA, including gene-specific DNA repair pathways, cellular accumulation and inactivation from thiol-containing reductants [e.g., glutathione (GSH) and metallothionein (MT)] belong among further pharmacological factors affecting antitumor effects of platinum compounds that do not operate directly at the level of DNA adducts.

Since the introduction of cisplatin, thousands of its bifunctional analogs have been synthesized and evaluated as po-

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ABBREVIATIONS: GSH, glutathione; MT, metallothionein; JM216, *cis,trans,cis*-[PtCl₂(CH₃COO)₂(NH₃)(cyclohexylamine)]; ESI, electrospray ionization; CT, calf thymus; bp, base pair(s); RT HIV-1, reverse transcriptase from human immunodeficiency virus type 1; CFE, cell-free extract; CHO, Chinese hamster ovary; NF-κB, nuclear factor-κB; HMG, high mobility group; FAAS, flameless atomic absorption spectrophotometry; *r*_b, the number of molecules of the platinum compound bound per nucleotide residue; CL, cross-link; *K*_{D(app)}, apparent dissociation constant; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; DPC, DNA-protein cross-linking coefficient; polyacrylamide gel electrophoresis; *r*_i, the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; NER, nucleotide excision repair.

common reagents used to synthesize adamplatin(II) and adamplatin(IV) were from Sigma.

The stock solutions of the platinum compounds were prepared at the concentration of 1×10^{-4} M in 10 mM NaClO₄. They were stored at 4°C in the dark, or for the experiments with cells, in water.

Calf thymus (CT) DNA (42% G + C; mean molecular mass 20,000 kDa) was also prepared and characterized as described previously (Brabec and Palecek, 1976). Plasmids pSP73 (2464 bp), pSP73KB (2455 bp), pUC19 (2686 bp), and pBR322 (4363 bp) were isolated according to standard procedures. The synthetic oligodeoxynucleotides (Fig. 1B) were purchased from VBC-Genomics (Vienna, Austria) and purified as described previously (Brabec et al., 1992; Kasparkova et al., 2000). In the present work, their molar concentrations are related to the whole duplexes. The duplexes containing central sequence TGGT/ACCA uniquely and site-specifically intrastrand cross-linked by adamplatin(II) or cisplatin were prepared and characterized as described previously (Malina et al., 2002). Expression and purification of the rat HMGB1 domain A (HMGB1a; residues 1–84) and HMGB1 domain B (HMGB1b; residues 85–180) were carried out as described previously (Stros, 2001). Restriction endonucleases, T4 polynucleotide kinase, and Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the 3'→5' proofreading domain KF[−]) were purchased from New England Biolabs (Beverly, MA). Reverse transcriptase from human immunodeficiency virus type 1 (RT HIV-1) was from Calbiochem (San Diego, CA). Riboprobe Gemini System II for transcription mapping containing T7 RNA polymerase was purchased from Promega (Madison, WI). Cell-free extracts (CFEs) were prepared from the HeLa S3 and CHO AA8 cell lines as described previously (Reardon et al., 1999). These extracts were kindly provided by J. T. Reardon and A. Sancar (University of North Carolina, Chapel Hill, NC). NF-κB (p50 dimer) was purchased from Active Motif (Rixensart, Belgium). GSH and ascorbic acid were purchased from Sigma, and rabbit MT-2 (Zn₇-MT-2) was a kind gift of Prof. M. Vasak (University of Zurich, Zurich, Switzerland). Acrylamide, bis(acrylamide), urea, thiourea, and NaCN were from Merck (Darmstadt, Germany). SDS was from Serva (Heidelberg, Germany). [γ -³²P]ATP was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Proteinase K was from Roche Diagnostics (Mannheim, Germany).

Measurements of Platinum Accumulation in A2780 and A2780cisR Cells. Ovarian carcinoma cell lines A2780 and A2780cisR were cultivated and treated as described in a previously published article (Kasparkova et al., 2003b). The cells were treated with cisplatin or adamplatin(IV) for 72 h at the concentrations corresponding to their IC₅₀ values in the cells tested (Kozubík et al., 2005), i.e., A2780 or A2780cisR cells were treated with cisplatin at the concentration of 1.34 or 24.23 μ M, respectively, and with adamplatin(IV) at the concentration of 0.22 or 1.40 μ M, respectively. The cells were analyzed for platinum by flameless atomic absorption spectrophotometry (FAAS). Standards and blank were prepared in the same way as the samples.

Transcription Mapping of DNA Adducts. Transcription of the (NdeI/HpaI) restriction fragment of pSP73KB DNA with DNA-dependent T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by Promega (Promega Protocols and Applications, 43-46, 1989–1990) and previously described in detail in Brabec and Leng (1993).

DNA Interstrand Cross-Linking. The amounts of interstrand cross-links (CLs) formed by adamplatin(II) in linear DNA were measured in pSP73 plasmid (2464 bp), which was first linearized by EcoRI (EcoRI cuts only once within pSP73 plasmid) and subsequently modified by adamplatin(II). The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions. Upon electrophoresis under denaturing conditions, 3' end-labeled strands of linearized pSP73 plasmid containing no interstrand CLs migrate as a 2464-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The radioactivity associated with the indi-

vidual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The frequency of interstrand CLs [the amount of interstrand CLs per one molecule of adamplatin(II) bound to DNA] was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. Other details of this assay can be found in previously published articles (Brabec and Leng, 1993; Brabec et al., 1999).

Gel Mobility Shift Assay Reactions with HMG Domain Proteins. The 5' end-labeled 20-bp oligonucleotide duplexes (TGGT shown in Fig. 1B), either unplatinated (controls) or containing the central platinum intrastrand CL in their top strands, were used, and their reaction with HMG domain proteins was performed and analyzed as described previously (He et al., 2000). The values of apparent dissociation constant, $K_{D(app)}$, were estimated in the manner described in He et al. (2000). Each $K_{D(app)}$ is the average of at least two measurements.

Inhibition of DNA Polymerization. We investigated in the present work DNA polymerization using the templates site-specifically modified by adamplatin(II) or cisplatin by DNA polymerase, namely, RT HIV-1. The 30-mer templates containing a single 1,2-GG intrastrand CL of adamplatin(II) or cisplatin were prepared in the same way as described in our recent work (Novakova et al., 2003, 2005). 17-mer DNA primer was complementary to the 3' termini of the 30-mer templates, respectively. The DNA substrates were formed by annealing templates and 5' end-labeled primers at a molar ratio of 3:1. Experiments were performed at 37°C in a volume of 50 μ l in a buffer containing 50 mM Tris.HCl, pH 7.4, 10 mM MgCl₂, 0.1 mM dithiothreitol, 50 μ g/ml bovine serum albumin, 0.1% Nonidet P-30, 100 μ M dATP, 100 μ M dCTP, 100 μ M dGTP, 100 μ M TTP, and 1.0 U of RT HIV-1. Reactions were terminated by the addition of EDTA, so that its resulting concentration was 20 μ M and by heating at 100°C for 30 s. Products were resolved by denaturing 24 or 15% PAA/8 M urea gel. Other details of these experiments can be found in our previously published articles (Novakova et al., 2003, 2005).

Repair Synthesis by Human Cell Extracts. Repair DNA synthesis of CFEs was assayed using pUC19 and pBR322 plasmids. Each reaction of 50 μ l contained 250 ng of nonmodified pBR322 and 250 ng of nonmodified or platinated pUC19, 2 mM ATP, 30 mM KCl, 0.5 mg/ml creatine phosphokinase (rabbit muscle), 20 mM each dGMP, dCTP, and TTP, 8 mM dATP, 74 kBq of [α -³²P]dAMP in the buffer composed of 40 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 0.5 mM dithiothreitol, 22 mM creatine phosphate, 1.4 mg/ml bovine serum albumin, and 150 μ g of CFE from the HeLa S3 cells. Reactions were incubated for 3 h at 25°C and terminated by adding EDTA to a final concentration of 20 mM, SDS to 0.6%, and proteinase K to 250 μ g/ml and then incubating for 30 min. The products were extracted with 1 volume phenol/chloroform (1:1). The DNA was precipitated from the aqueous layer by the addition of 1/50 volume of 5 M NaCl, 5 mg of glycogen, and 2.5 volumes of ethanol. After 20 min of incubation on dry ice and centrifugation at 12,000g for 30 min at 4°C, the pellet was washed with 0.5 ml of 70% ethanol and dried in a vacuum centrifuge. DNA was finally linearized before electrophoresis on a 1% agarose gel containing 0.3 mg/ml ethidium bromide.

Nucleotide Excision Assay. The 149-bp substrates containing a central, single 1,2-GG intrastrand CL of adamplatin(II) or cisplatin were assembled from three oligonucleotide duplexes as described in our previous work (Malina et al., 2002). The central duplex was TGGT(NER) duplex (Fig. 1B) to which two duplexes (arms) with random base pair sequences with overhangs partially overlapping those of the modified duplex were ligated (one to each side) by T4 DNA ligase. The top strand of the modified central duplexes were 5' end labeled with ³²P before ligation. Full-length substrates [non-modified, containing the 1,2-intrastrand CL of adamplatin(II) or cisplatin] were separated from unligated products on a denaturing 6% PAA gel, purified by electroelution, reannealed, and stored in annealing buffer (50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM

MgCl₂, and 1 mM dithiothreitol) at 20°C. In vitro repair of the 1,2-GG intrastrand CL of cisplatin or adamplatin(II) was measured in an excision assay as described previously (Malina et al., 2002) with minor modifications. The reaction mixtures (25 µl) contained 10 fmol of radiolabeled DNA, 50 µg of CFE from the HeLa S3 or CHO AA8 cell lines, 20 µM dATP, 20 µM dCTP, 20 µM dGTP, and 20 µM TTP in reaction buffer (23 mM HEPES, pH 7.9, 44 mM KCl, 4.8 mM MgCl₂, 0.16 mM EDTA, 0.52 mM dithiothreitol, 1.5 mM ATP, 5 µg of bovine serum albumin, and 2.5% glycerol) and were incubated at 30°C for 40 min. DNA was deproteinized and precipitated by ethanol. Reaction products were treated overnight with 0.4 M NaCN, pH 10 to 11, at 45°C and precipitated by ethanol before resolution on the gels. The excision products were separated on denaturing 10% PAA gels. The basic principles and other details of repair assays used in the present work can be found in our previously published article (Novakova et al., 2005).

Tritiated Thiourea Binding Assay. CT DNA at the concentration of 0.16 mg/ml was incubated with adamplatin(II) or cisplatin at various r_b values for 24 h. Of these solutions, 0.15 ml was added to 0.15 ml of 0.9 mM [³H]thiourea, prepared as described previously (Boudny et al., 1992), having specific radioactivity of 77 MBq/mmol. After 10 min incubation at 37°C, 0.8 ml of 0.15 M NaCl, pH 7.0, was added, and 1.0 ml of the resulting solution was layered on a nitrocellulose filter having pore diameters of 0.4 µm (Synpor VCHZ Syntezia, Pardubice, Czech Republic). To remove the unreacted thiourea, the filter was washed with 15 ml of 5% trichloroacetic acid. The filters were dried under an infrared lamp and then transferred to glass tubes to which 5 ml of toluene scintillator was added. The radioactivity was measured on a Wallac 1410 Beta spectrometer (PerkinElmer Wallac, Turku, Finland).

Ternary DNA-Protein Reactions. The 23-bp duplex(DP) (the central part of its nucleotide sequence shown in Fig. 1B corresponded to DNA consensus sequence of NF-κB) 5' end-labeled at its top strand was globally modified by the platinum complex at r_b , which corresponded to one platinum atom bound per duplex. Platinated DNA duplex(DP) (10 nM) was incubated with the various proteins (the molar ratio protein/duplex was 1:30) at 25°C for 1 h in the appropriate buffer: 10 mM Tris, pH 8, 10 mM EDTA, 0.1 µg of bovine serum albumin, 0.8% glycerol, and 2 mM MgSO₄ (reaction with KF⁻); 10 mM HEPES, pH 7.5, 10 mM MgCl₂, 50 mM LiCl, 100 mM NaCl, 1 mM spermidine, 0.2 mg/ml bovine serum albumin, and 0.05% Nonidet P-40 (reaction with HMGB1a); and 42 mM HEPES, 42 mM KCl, 1 mM MgCl₂, 0.02 mM EDTA, 2.5% glycerol, and 2% Ficoll (reaction with NF-κB). The reactions were analyzed by native gel electrophoresis (5%/10% SDS-PAGE; samples were loaded in cracking buffer without dithiothreitol). Quantification of free and adducted DNA was determined by using the bioimaging analyzer.

The efficiency of platinum complexes to form DNA-protein CLs examined in cells after their exposure in culture to these platinum compounds was examined by a potassium-SDS assay as described previously (Zhirkovich and Costa, 1992). The cell lines A2780 and A2780cisR were cultivated and treated for 48 h with the platinum complex in the same way as in the experiments focused on cellular uptake (vide supra). The DNA-protein cross-linking was evaluated using the DNA-protein cross-linking coefficient (DPC) defined as a ratio of the amount of SDS-precipitable DNA in treated cells to the amount of SDS-precipitable DNA in control cells (Zhirkovich and Costa, 1992). The values of DSC were normalized to the identical amount of platinum associated with the cells.

Reactions with Sulfur-Containing Compounds. Reactions of GSH and mammalian MT-2 with adamplatin(IV), adamplatin(II), and cisplatin were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH or MT-2 exactly as described in the previous work (Hagman et al., 2003); the absorbance at 260 nm reflects the presence of platinum-sulfur and disulfide bonds. The platinum compounds were mixed with GSH or MT-2 at 37°C in the medium of 10 mM NaClO₄ plus 0.1 mM phosphate buffer, pH 7.0, in the dark. Reactions were initiated by mixing

the platinum complex with the buffer followed by immediate addition of GSH or MT-2.

Reaction with Ascorbic Acid. The kinetic measurements of reactions of ascorbic acid with adamplatin(IV) were investigated by conventional absorption spectrophotometry monitoring a decrease of absorption in the range of 300 to 400 nm of solutions containing the platinum complex and ascorbic acid exactly as described in the previous work (Lemma et al., 2000). The platinum compound was mixed with ascorbic acid at 37°C in the medium of 0.04 M Tris-HClO₄ buffer, pH 7.0, in the dark. Reactions were initiated by mixing the platinum complex with the buffer followed by immediate addition of ascorbic acid.

Other Physical Methods. Absorption spectra were measured with a Beckman DU-7400 spectrophotometer (Beckman Coulter, Fullerton, CA). FAAS measurements were carried out with a Varian AA240Z Zeeman atomic absorption spectrometer (Varian, Inc., Palo Alto, CA) equipped with a GTA 120 graphite tube atomizer. For FAAS analysis, the cells were digested in nitric acid followed by the addition of H₂O₂ and HCl, whereas DNA was precipitated with ethanol and dissolved in 0.1 M HCl. Differential pulse polarography curves were recorded with the aid of a PARC electrochemical analyzer (model 384B; EG&G Berthold, Bad Wildbad, Germany). Purification of oligonucleotides with the aid of high-performance liquid chromatography was carried out on a Waters high-performance liquid chromatography system consisting of Waters 262 pump, Waters 2487 UV detector, and Waters 600S controller with MonoQ HR 5/5 column (Waters, Milford, MA). The gels were visualized by using the BAS 2500 FUJIFILM bioimaging analyzer (Fujifilm, Tokyo, Japan), and the radioactivities associated with bands were quantitated with the AIDA image analyzer software (Raytest, Straubenhardt, Germany).

Results

Cellular Platinum Complex Uptake. A possible explanation for the difference in cytotoxicity between cisplatin and the complexes containing alkylamine ligands with increased hydrophobicity, such as JM216 or adamplatin(IV) may be associated with differential cellular uptake of the compounds (Turanek et al., 2004). Cellular uptake of cisplatin and adamplatin(IV) was measured in A2780 and A2780cisR cells (sensitive and resistant to cisplatin, respectively) treated with the platinum compound for 72 h at the concentrations corresponding to the IC₅₀ values in the cells tested. These cancer cell lines sensitive and resistant to cisplatin, respectively, were among those used for testing activity of adamplatin(IV) in which this drug exhibited a markedly enhanced activity in comparison with cisplatin (IC₅₀ values found for cisplatin and adamplatin(IV) in A2780 cells were 1.34 and 0.22 µM, respectively, whereas these values found for cisplatin and adamplatin(IV) in A2780cisR cells were 24.23 and 1.40 µM, respectively (Kozubík et al., 2005). The results show that platinum associated with the cells treated with adamplatin(IV) was considerably higher than that with cisplatin (20 or 30 times in the sensitive and resistant cells, respectively; Table 1). Thus, the enhanced cellular uptake of adampla-

TABLE 1
Platinum uptake in cells

Table shows the uptake of cisplatin and adamplatin(IV) into A2780 and A2780cisR after 72 h. Each value (in picomoles of platinum per 10⁷ cells per micromolar) represents the average of six samples ± S.E..

	A2780	A2780cisR
Cisplatin	21 ± 2	3.7 ± 0.4
Adamplatin(IV)	422 ± 10	110 ± 5

tin(IV) seems to be an important factor responsible for enhanced cytotoxicity of this platinum complex in comparison with conventional cisplatin. In contrast, the uptake of adamplatatin(IV) in A2780 cells was ca. 4 times higher than that in A2780cisR cells, whereas the toxicity of adamplatatin(IV) in A2780 cells was ca. 6 times higher than that in A2780cisR cells. Hence, the cytotoxicity does not simply match the levels of cellular uptake, so that it is intuitively appealing to suggest that the cytotoxicity of adamplatatin(IV) may also be related to other pharmacological factors affecting platinum drug cytotoxicity.

DNA Modifications in Cell-Free Media. Platinum-DNA adducts induce a cellular signaling response regulating survival and apoptosis or necrosis. Platinum(IV) complexes undergo ligand substitution reactions that are slow relative to their platinum(II) counterparts. The antitumor activity of platinum(IV) complexes is therefore likely to require in vivo reduction to the kinetically more labile, and therefore reactive, platinum(II) congeners. Indeed, previous studies have shown that platinum(IV) metal centers are readily reduced by cellular components, such as small molecules involved in redox balance in the body (Hall and Hambley, 2002) to form the platinum(II) analogs that bind more rapidly to DNA. Thus, platinum(IV) compounds can be reduced extracellularly and enter the tumor cell as platinum(II) congener, or intracellularly having entered as platinum(IV) compound (Hall and Hambley, 2002). It implies that the administered adamplatatin(IV) serves as a prodrug for the chemotherapeutically active platinum(II) analog adamplatatin(II) (Fig. 1B), which is presumably the complex that binds to DNA (Brabec, 2002; Hall and Hambley, 2002). In the present study, we have applied some methodologies previously developed for cisplatin and its analogs to investigate the reaction products of adamplatatin(II) with DNA. Because this complex may be interesting from a clinical point of view, it is important to determine the extent to which it resembles the parent drug cisplatin in binding to its biological target(s).

Solutions of CT DNA at a concentration of 0.32 mg/ml were incubated with adamplatatin(II) at the r_i value in the range of 0.005 to 0.1 in 10 mM NaClO₄ at 37°C. r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA. At various time intervals, an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography for platinum not bound to DNA. The amount of platinum bound to DNA (r_b) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum present in the reaction. The amount of platinum coordinated to DNA increased with time with a rate identical to that found for cisplatin, and after approximately 24 h, adamplatatin(II) was quantitatively bound. It is noteworthy that the analytical method used monitors the covalent attachment of platinum complexes to DNA (so that the results are not affected by the subsequent closure of monofunctional adducts to bifunctional lesions) (Kim et al., 1990). pH of the reaction mixture containing DNA and adamplatatin was measured within 48 h after mixing DNA with the platinum complex, and no changes in pH were noticed.

Unless stated otherwise, the samples of DNA modified by adamplatatin(II) and cisplatin complexes, which were further analyzed in this work by biochemical or biophysical techniques, were prepared by incubating DNA with the platinum

complexes for 48 h (also see *Materials and Methods*). The binding experiments indicate that such platination reactions resulted in the coordination of all molecules of the platinum complexes, which made it possible to prepare easily and precisely the samples of DNA modified by the platinum complexes at a preselected value of r_b . It was also verified that the analyses of DNA samples modified by adamplatatin(II) by using biochemical and biophysical techniques described in this work yielded identical results if aged or fresh solutions of platinum complexes were used.

In vitro RNA synthesis by RNA polymerases on DNA templates containing several types of bifunctional adducts of platinum complexes can be prematurely terminated at the level or in the proximity of adducts (Brabec and Leng, 1993). Cutting of pSP73KB DNA (Brabec and Leng, 1993) by NdeI and HpaI restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 2B). This fragment contained T7 RNA polymerase promoter (in the upper strand close to its 3' end; Fig. 2B). The

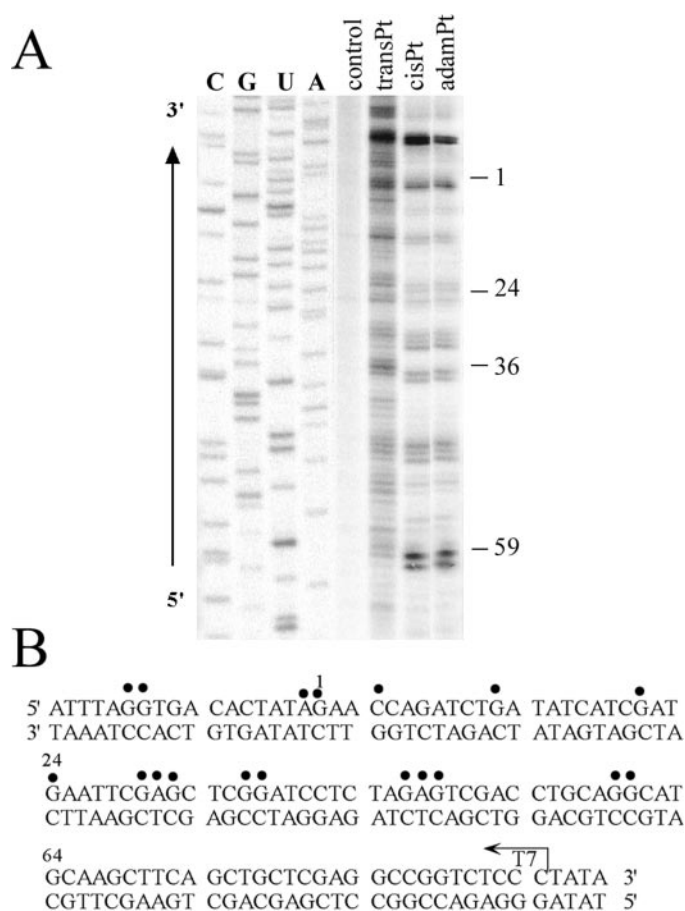


Fig. 2. Inhibition of RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by platinum complexes. A, autoradiograms of 6% PAA/8 M urea sequencing gels showing inhibition of RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment containing adducts of platinum complexes. Lanes: C, G, U, and A, chain-terminated marker RNAs; control, unmodified template; transPt, cisPt, and adamPt, template modified by transplatin, cisplatin, or adamplatatin(II) at $r_b = 0.01$, respectively. B, Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by platinum complexes. The arrow indicates the start of the T7 RNA polymerase, which was used as template the upper strand of NdeI/HpaI fragment of pSP73KB DNA. The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

experiments were carried out using this linear DNA fragment, modified by cisplatin and adamplatin(II) at $r_b = 0.005$, for RNA synthesis by T7 RNA polymerase (Fig. 2, lanes cisPt and adamPt). RNA synthesis on the template modified by the platinum complexes yielded fragments of defined sizes, which indicates that RNA synthesis on these templates was prematurely terminated (Fig. 2A). The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of cisplatin and adamplatin(II) were identical and occurred at guanine (G) sites and to a considerably lesser extent at adenine (A) sites. These G and A sites were mostly contained in GG or AG sites, which are preferential DNA binding sites for conventional cisplatin. Taken together, the results of the transcription mapping experiments suggest that base sequence selectivity of cisplatin and adamplatin(II) are similar and that also the major adducts formed on DNA by adamplatin(II) and cisplatin are identical, presumably 1,2-GG or AG intrastrand CLs.

Cisplatin also forms in DNA a small amount of interstrand CLs. Despite their low abundance, these lesions are also considered relevant to the antitumor effects of cisplatin (Brabec, 2000). The amounts of interstrand CLs formed by adamplatin(II) in linear DNA were measured in linearized pSP73 plasmid, which was modified by adamplatin(II) to various values of r_b . The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions (Brabec and Leng, 1993; Brabec et al., 1999). The bands corresponding to more slowly migrating interstrand cross-linked fragments were noticed if adamplatin(II) was used to modify linearized DNA at r_b as low as 5×10^{-4} (Fig. 3). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The frequency of interstrand CLs produced by adamplatin(II) was somewhat lower ($\sim 4\%$) than cross-linking efficiency of cisplatin (6%; Brabec and Leng, 1993).

Recognition of Major 1,2-GG Intrastrand DNA Adducts by HMG-Domain Proteins, Translesion DNA Synthesis across These Adducts, and Their Repair. An important feature of the mechanism of action of cisplatin is that the major adducts of this platinum drug (1,2-GG and AG

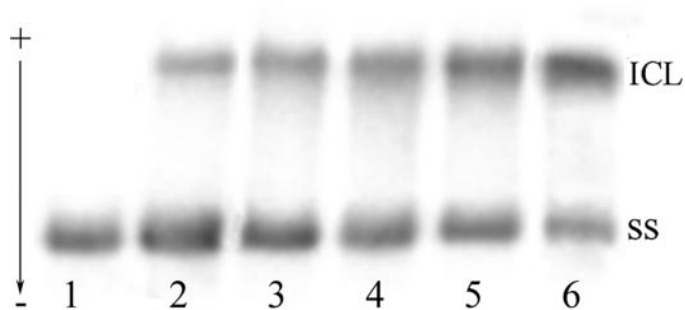


Fig. 3. Formation of the interstrand CLs by adamplatin(II) in pSP73 plasmid linearized by EcoRI. Autoradiogram of denaturing 1% agarose gels of linearized DNA, which was 3' end labeled. The interstrand cross-linked DNA occurs as the top bands (marked as ICL) migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands and marked as ss). The fragment was nonplatinated (control) (lane 1) or modified by adamplatin(II) at $r_b = 5 \times 10^{-4}$, 1×10^{-3} , 2×10^{-3} , 4×10^{-3} , or 6×10^{-3} (lanes 2–6, respectively).

intrastrand CLs) are recognized by proteins containing HMG domains (Brabec, 2002). It has also been shown (Brabec, 2002) that the binding of these proteins to cisplatinated DNA mediates the antitumor effects of this platinum drug. In addition, the replacement of one or both ammine groups in cisplatin by various, mostly cyclic nonleaving ligands, markedly affected affinity of HMG-box proteins to the 1,2-GG intrastrand CLs (Wei et al., 2001). Hence, it was of great interest also to examine whether the replacement of the ammine group in cisplatin by 1-adamantylamine could also affect affinity of HMG-box proteins to this major adduct.

The interactions of the rat HMGB1a (residues 1–84) and HMGB1b (residues 85–180) with the 1,2-GG intrastrand CLs of adamplatin(II) were investigated using gel mobility shift assay. In these experiments, the 20-bp duplex TGGT (Fig. 1B) was modified so that it contained a single, site-specific 1,2-GG intrastrand CL of adamplatin(II) or cisplatin. Because adamplatin(II) contains two different nonleaving ligands, two orientational isomers (5' and 3') could be formed similarly as in the case of 1,2-GG intrastrand CL of *cis*-[PtCl₂(NH₃)(cyclohexylamine)], which is a major metabolite of JM216 (Hartwig and Lippard, 1992). The reactions of all oligonucleotides containing the central GG sequences (the top strand in the duplex TGGT in Fig. 1B) with adamplatin(II) gave one product as revealed by ion exchange fast-performance liquid chromatography and 24% PAA/8 M urea gel electrophoresis. It is possible that only one orientational isomer was formed in these reactions. Two orientational isomers could have been formed, but they could not be resolved by fast-performance liquid chromatography or gel electrophoresis.

The binding of the HMGB1a and HMGB1b to the DNA probes containing the 1,2-GG intrastrand CL of adamplatin(II) was detected by retardation of the migration of the radiolabeled 20-bp probes through the gel (He et al., 2000) (Fig. 4). HMGB1a and HMGB1b exhibited negligible binding to the unmodified 20-bp duplexes. As indicated by the pres-

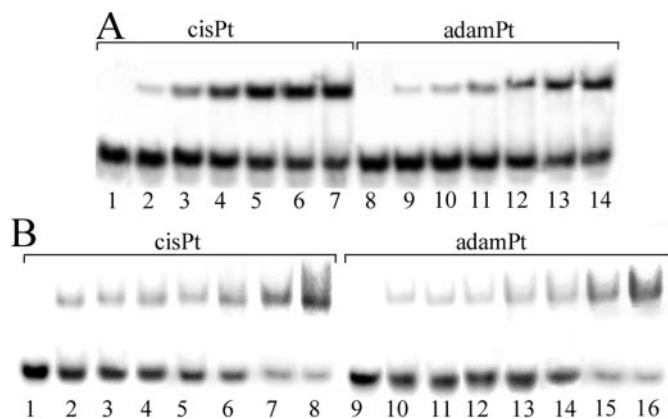


Fig. 4. Analysis of the binding affinity of 20-bp DNA containing a single, site-specific 1,2-GG intrastrand CL of cisplatin or adamplatin(II) for HMGB1a (A) and HMGB1b (B) on 6% polyacrylamide gel. Lanes in A: lanes 1 to 7, duplex containing the CL of cisplatin; lanes 8 to 14, duplex containing the CL of adamplatin(II); lanes 1 and 8, no protein added; lanes 2 and 9, 0.5 nM; lanes 3 and 10, 1.0 nM; lanes 4 and 11, 5.0 nM; lanes 5 and 12, 10 nM; lanes 6 and 13, 30 nM; and lanes 7 and 14, 60 nM HMGB1a. Lanes in B: lanes 1 to 8, duplex containing the CL of cisplatin; lanes 9 to 16, duplex containing the CL of adamplatin(II); lanes 1 and 9, no protein added; lanes 2 and 10, 10 nM; lanes 3 and 11, 20 nM; lanes 4 and 12, 50 nM; lanes 5 and 13, 0.1 μ M; lanes 6 and 14, 0.2 μ M; lanes 7 and 15, 0.5 μ M; and lanes 8 and 16, 1.0 μ M HMGB1b.

ence of a shifted band whose intensity increases with increasing protein concentration, both HMGB1a and HMGB1b recognize the duplex containing the 1,2-GG intrastrand CL of adamplatin(II) (Fig. 4). These data indicate that HMGB1a binds the probe containing the 1,2-GG intrastrand CL of adamplatin(II) with a relatively high affinity, although somewhat lower than to the probe containing the same adduct of cisplatin (Fig. 4A). Evaluations of these titration data afforded $K_{D(\text{app})}$ values of 4.0×10^{-8} and 5.2×10^{-8} M for the binding of HMGB1a to the adducts of cisplatin and adamplatin(II), respectively. The titration of the 20-bp duplex containing the 1,2-GG intrastrand CL of adamplatin(II) with HMGB1b has revealed (Fig. 4B) that this protein also binds to the 1,2-GG intrastrand CL of adamplatin(II) with a similar affinity as to the probe containing 1,2-GG intrastrand CL of cisplatin. Evaluations of these titration data afforded $K_{D(\text{app})}$ values of 3.8×10^{-7} and 4.0×10^{-7} M for the binding of HMGB1a to the adducts of cisplatin and adamplatin(II), respectively. The affinity of HMGB1b to these platinum lesions was, however, considerably smaller than that of HMGB1a as expected (Wei et al., 2001; Malina et al., 2002). Hence, it is reasonable to expect that the full-length HMGB1 protein, which is an abundant chromosomal protein in mammalian cells and the candidate protein for participation in the repair shielding mechanism of antitumor activity of cisplatin (Zamble and Lippard, 1999; Brabec, 2002), has a similar affinity to the 1,2-GG intrastrand CL of adamplatin(II).

DNA secondary structures have significant effects on processivity of a number of prokaryotic, eukaryotic, and viral DNA polymerases. The character of DNA templates containing site-specific platinum adducts dictates whether DNA polymerases are blocked or can traverse through the lesion. DNA polymerization using the templates site-specifically modified by 1,2-GG intrastrand CLs of adamplatin(II) or cisplatin by RT HIV-1 was investigated to reveal the potential differences imposed on DNA by these two adducts. We constructed the 17-mer/30-mer primer-template duplexes TGGT (Fig. 5A) unplatinated or containing the 1,2-GG intrastrand CL of either cisplatin or adamplatin(II). The first 17 nucleotides on the 3' terminus of the 30-mer template strand were complementary to the nucleotides of the 17-mer primer, and the 3' guanine involved in the 1,2-GG CL of cisplatin or adamplatin(II) on the template strand was located at its 20th position from the 3' terminus (Fig. 5A). After annealing the 17-nucleotide primer to the 3' terminus of the unplatinated or platinated template strand (positioning the 3' end of the primer three bases before the adduct in the template strand), we examined DNA polymerization through the unique CLs on the template by RT HIV-1 in the presence of all four deoxyribonucleoside 5'-triphosphates. The reaction was stopped at various time intervals, and the products were analyzed using a sequencing gel (Fig. 5A). Polymerization using the template containing the CL of cisplatin proceeded rapidly up to the nucleotides at the sites opposite the CL, such that the 20- and 21-nucleotide products accumulated to a significant extent (Fig. 5A, lanes 6–10). There was no accumulation of DNA intermediate products with the 30-mer control template as the full-length product was being formed (Fig. 5A, lanes 1–5). The full-length products were also noticed with the 30-mer template containing the CL of cisplatin, although in a smaller amount. The results are in agreement with previously published work (Marini et al., 2005)

and confirm that the 1,2-GG intrastrand CL of cisplatin inhibits DNA synthesis, but translesion synthesis may occur. In contrast, under the same experimental conditions, DNA polymerization by RT HIV-1 on the template containing 1,2-GG intrastrand CL of adamplatin(II) (Fig. 5A, lanes 11–15) proceeded up to the site preceding the platinated GG sequence so that also the 19-nucleotide product accumulated to a significant extent and then also the 20-nucleotide product accumulated corresponding to the nucleotide at the site opposite the 5' G involved in the CL. There was almost no accumulation of shorter and longer DNA intermediates, and importantly, fewer full-length products accumulated than when DNA polymerization using the template site-specifically modified by 1,2-GG intrastrand CLs of cisplatin was examined (Fig. 5B). This result indicates that the 1,2-GG intrastrand CLs of adamplatin(II) impede elongation of DNA to a greater extent than the major adducts of cisplatin (Fig. 5B).

The high degree of structural and sequence conservation of the domains among eukaryotic, prokaryotic, and viral polymerases suggests that the results from studies of the RT HIV-1 should also be applicable to other DNA polymerases. Hence, the stronger inhibition of DNA polymerization by the adamplatin(II) 1,2-GG intrastrand CL reflects an important

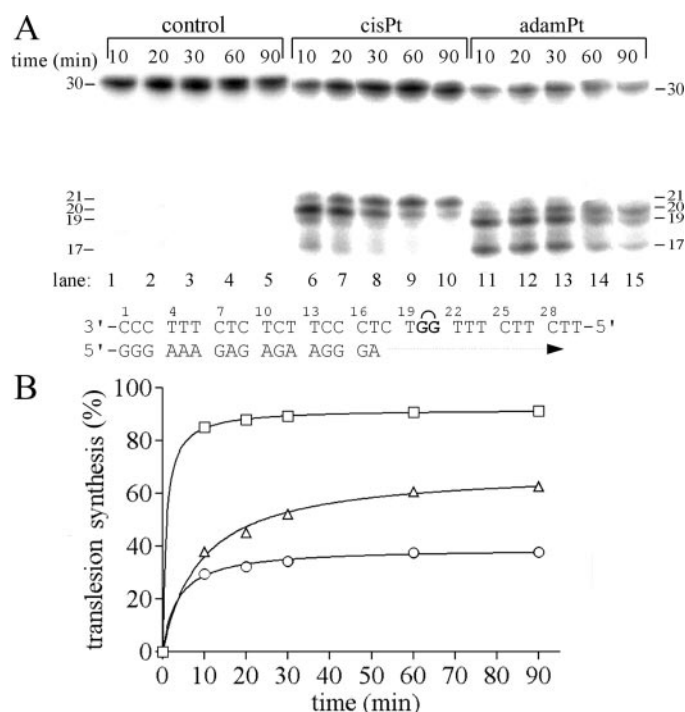


Fig. 5. Primer extension activity of RT HIV-1. **A**, experiments were conducted using the 17-mer/30-mer primer/template duplex for various times using undamaged template (lanes 1–5), the template containing 1,2-GG intrastrand CL of cisplatin (lanes 6–10), or adamplatin(II) (lanes 11–15). Timings were as follows: 10 min, lanes 1, 6, and 11; 20 min, lanes 2, 7, and 12; 30 min, lanes 3, 8, and 13; 60 min, lanes 4, 9, and 14; and 90 min, lanes 5, 10, and 15. The pause sites opposite the platinated guanines are marked 19 and 20. The pause sites opposite the nucleotides 17 and 21 of the template are marked 17 and 21, respectively. The nucleotide sequence of the primer and the templates including its nucleotide numbering are shown beneath the gel. **B**, The time dependence of the inhibition of DNA synthesis on undamaged (control) template (squares), DNA containing 1,2-GG intrastrand CL of cisplatin (triangles), and DNA containing 1,2-GG intrastrand CL of adamplatin(II) (circles) is indicated. Data are means \pm S.E. from three different experiments with two independent template preparations.

difference in biological processes of replication or DNA repair in comparison with the major adduct of cisplatin.

Figure 6 illustrates an experiment that measures DNA repair synthesis by a repair-proficient HeLa CFE in pUC19 plasmid modified at $r_b = 0.05$ by adamplatin(II) or cisplatin. Repair activity was monitored by measuring the amount of incorporated radiolabeled nucleotide. A similar amount of undamaged pBR322 of a slightly different size is included in the reactions to show the background incorporation into undamaged plasmid. This background incorporation was subtracted from that found for metallated pUC19 plasmid. Different levels of damage-induced DNA repair synthesis were detected in the plasmid modified by adamplatin(II) and cisplatin (Fig. 6B). The level of the synthesis detected in the plasmid modified by adamplatin(II) was by $\sim 25\%$ lower than that in the plasmid modified by cisplatin.

DNA repair synthesis can be due to various DNA repair mechanisms. Bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics, including cisplatin, are removed from DNA by nucleotide excision repair (NER), which is an important component of the mechanism underlying biological effects of these agents. Efficient removal of CLs formed in DNA by platinum antitumor compounds has already been reported for various NER systems, including human and rodent excinucleases (Zehnulova et al., 2001; Kasparkova et al., 2003a). The result presented in Fig. 7A, lane 4, is consistent with these reports. The major excision fragment contains 28 nucleotides, and other primary excision fragments are 24–29 nucleotides (Reardon et al., 1999; Zehnulova et al., 2001; Kasparkova et al., 2003a). The 1,2-GG intrastrand CL of adamplatin(II) was also excised by both human and rodent excinucleases (shown for rodent excinuclease in Fig. 7A, lane 6), although with a lower efficiency than the major intrastrand CL of cisplatin. Thus, these results demonstrate the lower efficiency (by ca. 20%) of the mammalian NER systems used in the present work to excise major intrastrand adducts of adamplatin(II) (Fig. 7B).

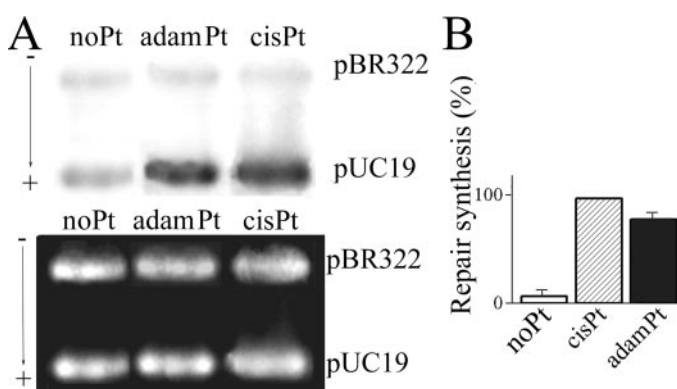


Fig. 6. In vitro repair synthesis assay of the extract prepared from the repair-proficient HeLa cell line. Repair synthesis used as substrates nonmodified pBR322 plasmid and pUC19 plasmid nonmodified (lane noPt) or modified at $r_b = 0.05$ by cisplatin (lane cisPt), or adamplatin(II) (lane adamPt). A, Results of a typical experiment. Top, autoradiogram of the gel showing incorporation of [α - 32 P]dCMP. Bottom, photograph of the ethidium bromide-stained gel. B, incorporation of dCMP into nonmodified, platinated, or platinated plasmids. For all quantifications representing mean values of three separate experiments, incorporation of radioactive material is corrected for the relative DNA content in each band. The radioactivity associated with the incorporation of [α - 32 P]dCMP into DNA modified by cisplatin was taken as 100%.

DNA-Protein Cross-Linking. When we isolated and purified the short oligonucleotide duplexes containing single, site-specific 1,2-GG intrastrand adduct of adamplatin(II) or cisplatin as DNA probe for the studies of affinity of HMG-domain proteins to this adduct (vide supra), we noticed that the intrastrand CL was formed by adamplatin(II) noticeably more slowly, although the overall level of the oligonucleotide platination was similar. A reasonable explanation of this observation is that adamplatin(II) forms on DNA more monofunctional adducts that close to bifunctional CL less readily than monofunctional adducts of cisplatin. Thus, the monofunctional adducts of adamplatin(II) might persist for a considerably longer time than the same adducts of cisplatin. To test this hypothesis, CT DNA was incubated in 10 mM NaClO₄ at 37°C for 24 h with adamplatin(II) or cisplatin so that the level of platination corresponded to r_b values of 0.01, 0.05, and 0.1. We trapped the reactive monofunctional adducts by fixing radioactive (tritiated) thiourea on the platinum-DNA complex (Boudny et al., 1992; Brabec et al., 1996). The intriguing observation was that the radioactivity associated with DNA treated with adamplatin(II) was more than 3 times higher than that with cisplatin, confirming that adamplatin(II) forms reactive monofunctional adducts with pronouncedly higher frequency than cisplatin.

Adamplatin(II) and cisplatin were investigated for their ability to form ternary DNA-protein complexes. The 23-bp duplex(DP) (the central part of its nucleotide sequence shown in Fig. 1B corresponded to DNA consensus sequence of NF- κ B) 5' end-labeled at its top strand was globally modified by adamplatin(II) or cisplatin for 24 h to the r_b value that corresponded to 0.5 platinum atom bound per duplex. The duplex modified by adamplatin(II) or 10 nM cisplatin was mixed with HMGB1a, KF⁻, or NF- κ B (p50 dimer) proteins (the molar ratio protein/duplex was 30, 1, and 5, respectively). The different molar ratios were used to reflect the different binding affinity [$K_{D(app)}$] of the proteins to the platinated duplex. Ternary DNA-platinum-protein cross-linking efficiency was assessed by SDS-PAGE shift assay. These proteins were chosen as representatives of DNA-binding proteins with structural, enzymatic, or regulation function. Fractions were detected by SDS-PAGE with significantly retarded mobility (Fig. 8, lanes 6–8 and 10–12) compared with that of the free probe (Fig. 8, lanes 1, 5, and 9). These more slowly migrating fractions were eliminated after treatment with NaCN or proteinase K converting them to those of the unmodified probes (data not shown). These results suggest that the species is a protein-DNA CL tethered by platinum-DNA and platinum-protein covalent bonds. Although the proteinase K and NaCN experiments clearly indicate that protein is the species cross-linked to DNA, the amino acids participating in the cross-linking reaction have not been determined. It is noteworthy that the amount of radioactivity associated with the bands corresponding to DNA-protein CLs formed by adamplatin(II) was 6 to 7 times higher than that by cisplatin, demonstrating that adamplatin(II) exhibits a considerably higher efficiency to form ternary DNA-platinum-protein CLs than cisplatin.

We have also determined the efficiency of adamplatin(IV) and cisplatin to form DNA-protein CLs in A2780 and A2780cisR cells after their exposure in culture to these platinum drugs using a potassium-SDS assay (Zhitkovich and Costa, 1992). The cells were treated with adamplatin(IV) or

cisplatin at the concentrations corresponding to the IC_{50} values in the cells tested (vide supra) for 72 h. The DNA-protein cross-linking was evaluated using DPC (Zhitkovich and Costa, 1992). Because the cellular uptake of cisplatin and adamplatin(IV) is different (vide supra), the values of DPC were normalized to the identical amount of platinum associated with the cells. Exposure of A2780 and A2780cisR cells to adamplatin(IV) resulted in the formation of substantial amounts of DNA-protein CLs (Table 2). The efficiency of adamplatin(IV) in sensitive and resistant cells was 4 and 9 times higher, respectively, than that found for the exposure of these cells to cisplatin.

Reactions with Sulfur-Containing Compounds and Ascorbic Acid. Platinum(II) or platinum(IV) compounds have a strong thermodynamic preference for binding to sulfur donor ligands. Hence, before antitumor platinum drugs reach DNA in the nucleus of tumor cells, they may interact with various compounds, including sulfur-containing molecules. These interactions are generally thought to play a role in mechanisms underlying tumor resistance to platinum compounds, their inactivation, and their side effects (Reedijk, 1999). Therefore, interest in the interactions of platinum antitumor drugs with sulfur-containing molecules of biological significance has recently increased markedly.

Examples of endogenous thiols to which platinum complexes bind when they are administered intravenously or after they enter the cell are GSH and MT. MTs are small (~6- to 7-kDa) cellular metalloproteins that strongly bind metal ions. The metal ion binding domains of MT-2 consist of 20 cysteine residues arranged in two thiol (S)-rich sites (Romero-Isart and Vasak, 2002). In the present work, we investigated reactions of GSH and mammalian MT-2 with adamplatin(IV), adamplatin(II), and cisplatin using UV-absorption spectrophotometry (Hagman et al., 2003).

Adamplatin(IV), adamplatin(II), or cisplatin at a concentration of 33 μ M was mixed with 5 mM GSH (this concentration of GSH is physiologically relevant; Romero-Isart and Vasak, 2002) at 37°C in the medium of 10 mM $NaClO_4$ plus 10 mM Tris-HCl buffer, pH 7.4. The half-times of the reactions, which mainly result in the formation of platinum-sulfur bonds, were 10 and 113 min for reactions of adamplatin(IV) and adamplatin(II) with GSH, respectively. Cisplatin reacted with GSH with the half-time of 11 min [i.e., with a similar rate to that of adamplatin(IV) but faster than adamplatin(II)].

Adamplatin(II) or cisplatin at the concentration of 200 μ M were also mixed with MT-2 at the concentration of 2.1 μ M under the same conditions. The half-times of the reaction of adamplatin(II) or cisplatin with MT-2 were similar (42 or 40 min, respectively). In contrast, no changes in the absorbance at 260 nm, indicating reaction of MT-2 with adamplatin(IV), were noticed if MT-2 at the concentration as high as 200 μ M was incubated with 200 μ M adamplatin(IV) for 1 week.

The anticancer activity of adamplatin(IV) is likely to be due to its reduction to more reactive platinum(II) analog in agreement with the assumption that these compounds act as prodrugs to their platinum(II) analogs. Hence, reduction of adamplatin(IV) by potential bioreductant such as ascorbic acid may be also important for understanding of the mechanism of antitumor activity of this platinum complex (Lemma et al., 2000). Adamplatin(IV) at the concentration of 0.15 mM was mixed with 1.5 mM ascorbic acid (this concentration of ascorbic acid is physiologically relevant; Lemma et al., 2000). The half-time of the reaction was found 15 min. Thus, reduction of adamplatin(IV) is fairly rapid similarly as reduction of JM216 by ascorbic acid (Lemma et al., 2000) compared with hydrolytic biotransformation pathways.

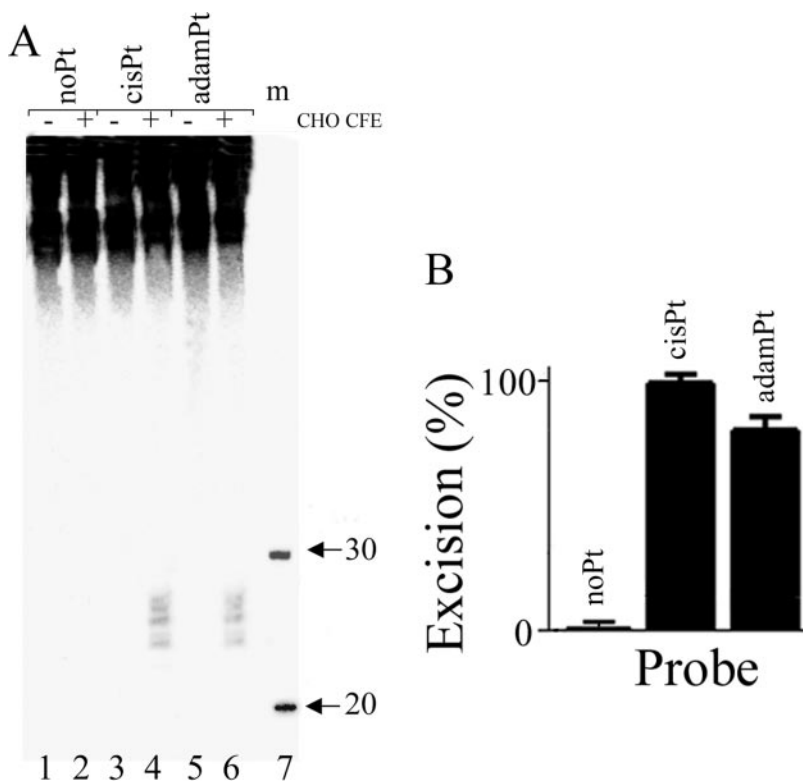


Fig. 7. Excision of the intrastrand CLs of platinum complexes by rodent excinuclease. A, substrates were incubated with CHO AA8 CFE and subsequently treated overnight with NaCN before analysis in 10% PAA/8 M urea denaturing gel. Lanes 1 and 2, control, unplatinated substrate; lanes 3 and 4, the substrate containing the 1,2-GG intrastrand CL of cisplatin; lanes 5 and 6, the substrate containing the 1,2-GG intrastrand CL of adamplatin(II); lanes 1, 3, and 5, no extract added; lanes 2, 4, and 6, the substrates were incubated with CHO AA8 CFE for 40 min at 30°C. Lane 7, the 20- and 30-nucleotide markers. B, quantitative analysis of removal of the adducts. The columns marked as noPt, cisPt, and adamPt represent unplatinated substrate, the substrate containing 1,2-GG intrastrand CL of cisplatin, or 1,2-GG intrastrand CL of adamplatin(II), respectively. The radioactivity associated with the fragments excised from the duplex containing the 1,2-GG intrastrand CL of cisplatin was taken as 100%. Data are the average of two independent experiments done under the same conditions; bars indicate range of excision.

Discussion

Adamplatin(IV) proved to be quite toxic to the tumor cells, with IC₅₀ values that were markedly lower than those observed for cisplatin and JM216 (Turanek et al., 2004; Kozubik et al., 2005). The origin of this effect may be multifactorial. The major pharmacological factors affecting platinum drug cytotoxicity are cellular uptake and efflux; structure; and frequency of target (DNA) adducts, including their intracellular processing; and metabolic deactivation by sulfur nucleophiles (Brabec and Kasparkova, 2005). All these factors combine to affect signaling pathways, leading to apoptosis or necrosis of tumor cells.

The present study reveals that the cellular uptake of adamplatin(IV) is considerably greater than that of cisplatin and JM216 (O'Neill et al., 1999). An increase of hydrophobicity of ligands of various platinum complexes has been shown to positively correlate with enhanced cellular uptake (Moeller et al., 2000). Thus, the observation that the cellular uptake of adamplatin(IV) is greater than that of the closely related JM216 is consistent with a considerably more hydrophobic character of alkylamine ligands in adamplatin(IV) (Turanek et al., 2004). The cellular accumulation was enhanced in A2780cisR cells resistant to cisplatin over sensitive A2780 cells, suggesting a mechanism for enhancement of tumor cell selectivity toward cisplatin-resistant cells. How-

ever, there is no clear correlation between uptake and cytotoxicity of adamplatin(IV) within the tumor cells sensitive and resistant to cisplatin. Nevertheless, the results confirm the remarkable uptake of adamplatin(IV) so that at least part of the increased cytotoxicity of adamplatin(IV) over cisplatin seems to be attributable to an increased intracellular accumulation.

It is widely held that reduction to platinum(II) is essential for the anticancer activity of platinum(IV) complexes (Hall and Hambley, 2002), so that adamplatin(II) (the analog of cisplatin) is presumably the complex that binds to DNA. The adducts of conventional cisplatin distort DNA conformation, inhibit replication and transcription (but they are also bypassed by DNA or RNA polymerases), and trigger apoptosis or necrosis (Fuentes et al., 2003). In addition, cisplatin adducts are removed from DNA mainly by NER. They are, however, also recognized by several proteins, such as HMG-domain proteins. The details of how the binding of HMG-domain proteins to cisplatin-modified DNA sensitizes tumor cells to cisplatin are still not completely resolved, but possibilities such as shielding cisplatin-DNA adducts from excision repair or these proteins being titrated away from their transcriptional regulatory function have been suggested as clues for how they are involved in antitumor activity (Brabec, 2002). The ability of HMGB1 protein, and probably other cisplatin-DNA-binding proteins, to influence the efficacy of the drug may be dependent on tumor cell type (Wei et al., 2003).

In broad terms, we have demonstrated that the DNA binding mode of adamplatin(II), including sequence preference, type of the major adducts, resulting conformational alterations, and their recognition by HMG-domain proteins, is not very different from that of cisplatin. In contrast, there are pronounced differences in several factors associated with the DNA binding mode, which may be associated with different biological effects of adamplatin(IV) and cisplatin.

The major adducts of the metabolite of adamplatin(IV) can inhibit DNA polymerization to a limited extent (although more efficiently than the same adducts of cisplatin), so that they can also be bypassed by DNA polymerases (Fig. 5). Likewise, the 1,2-GG intrastrand CLs of adamplatin(II) are removed from DNA by the components of mammalian NER system present in CFEs with lower efficiency than the CLs of cisplatin (Fig. 7), so that it is reasonable to expect that they may persist much longer on DNA than the adducts of cisplatin. Thus, the observation that the major adducts of adamplatin(II) impede elongation of DNA to a greater extent than those of cisplatin and that these adducts of adamplatin(II) are removed from DNA less easily may imply that these pharmacological factors are at least partially responsible for altered antitumor effects of this new platinum(IV) drug in comparison with conventional cisplatin.

The results of the present work also demonstrate that the monofunctional adducts of adamplatin(II) (in cell-free media) and adamplatin(IV) (in cells) readily cross-link proteins, apparently owing to the slow chelation reaction of monofunctional adducts of adamplatin(II). It is noteworthy that earlier observations have demonstrated that cisplatin also forms DNA-protein ternary CLs (Zhitzkovich and Costa, 1992). However, the monofunctional adducts of cisplatin formed in the first step of the reaction with DNA close to bifunctional CLs with a relatively fast rate so that these adducts do not

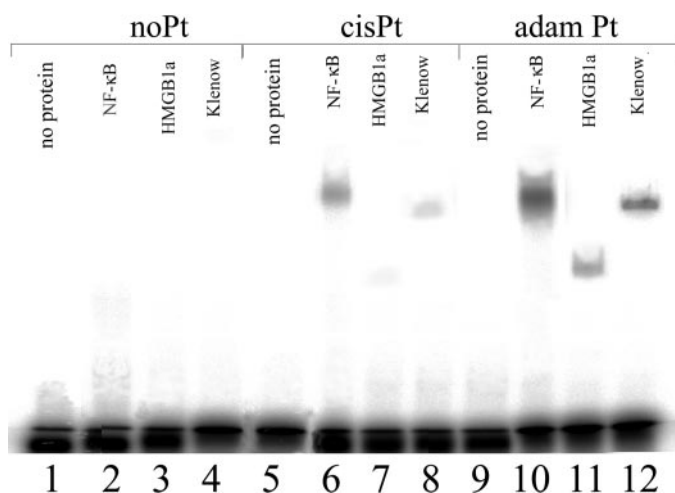


Fig. 8. DNA-protein cross-linking. Ternary complex formation of unmodified (lanes 1–4) and platinated oligodeoxyribonucleotide duplex (DP) containing single, site-specific 1,2-GG intrastrand CL of cisplatin (lanes 5–8) or adamplatin(II) (lanes 9–12); no protein added (lanes 1, 5, and 9), NF-κB added (lanes 2, 6, and 10), HMGB1a added (lanes 3, 7, and 11), KF[−] added (lanes 4, 8, and 12). Ternary complex formation was assessed by SDS-PAA gel electrophoresis. The nucleotide sequence of the duplex(DP) is shown in Fig. 1B.

TABLE 2

DNA-protein cross-linking in cells

DNA-protein CL formation following exposure of A2780 and A2780cisR cells for 48 h to cisplatin or adamplatin(IV). After washing, cells were subjected to the potassium-SDS procedure following overnight storage at −70°C. The cross-linking was evaluated using the DPC defined as a ratio of the amount of SDS-precipitable DNA in treated cells to the amount of SDS-precipitable DNA in control cells (Zhitzkovich and Costa, 1992). The values of DPC shown are those normalized to the identical amount of platinum associated with the cells. Each value of DPC represents the average of four samples ± S.E. For other details, see the text.

	A2780	A2780cisR
Cisplatin	127 ± 7	53 ± 7
Adamplatin(IV)	508 ± 10	477 ± 12

persist for long enough to allow their extensive cross-linking to proteins to occur (Bancroft et al., 1990). Consistent with this conclusion is the relatively very low frequency of the DNA-protein ternary CLs produced in Chinese hamster ovary cells treated with cisplatin (<1%) (Plooy et al., 1984), although other studies revealed somewhat higher amounts of these ternary lesions in other types of tumor cells (Farrell et al., 2004). Adamplatin(II) forms more monofunctional adducts on DNA than cisplatin as a result of retardation of the rearrangement of its monofunctional to bifunctional adducts and favorable competitive reaction with protein. It is noteworthy that a decrease in this chelation rate was already reported for other cisplatin analogs bearing bulky hydrophobic nonleaving amine ligands, such as *trans*-1,2-diaminocyclohexanedichloroplatinum(II) (Page et al., 1990) or *cis*-[*N*-2-amino-*N*-2-methylamino-2,2,1-bicycloheptane]dichloroplatinum(II) (Lambert et al., 1995), and is due to incorporation of steric hindrance by bulky hydrophobic amine ligand (Lambert et al., 1995). In aggregate, the capacity of DNA adducts of adamplatin(II) to cross-link proteins supports the idea that the capability of DNA adducts of this platinum compound to cross-link proteins represents an important feature of the mechanism underlying antitumor effects of this new platinum(IV) compound.

Other details of the mechanism of the DNA-protein cross-linking by adamplatin(IV) or adamplatin(II) cross-linking are unknown. However, this cross-linking could result from a substitution of the labile ligand by a nucleophilic group of the protein such as a histidine, imidazole, or more likely a thioether group of a methionine or a thiol group of a cysteine, within the protein-DNA recognition complex.

The earlier reports have demonstrated that cross-linking proteins to platinum-DNA adducts markedly enhances the efficiency of these adducts to terminate DNA polymerization by DNA polymerases in vitro and to inhibit removal of these adducts from DNA by NER (Novakova et al., 2003). Hence, it is reasonable to suggest that DNA-protein ternary CLs produced by adamplatin could persist considerably longer than the non-cross-linked adducts, potentiating the toxicity of adamplatin(IV) toward tumor cells sensitive to this drug. Thus, covalent cross-linking of DNA and proteins by adamplatin represents a potential novel mechanism through which this compound could exert its antitumor activity.

The results of the present work also demonstrate that adamplatin(IV) reacts with GSH with the rate similar to that of parent cisplatin. In contrast, this new platinum(IV) compound is radically more potent also in the tumor cell line A2780cisR resistant to cisplatin (Kozubík et al., 2005), which is known to be resistant also through elevated levels of reduced GSH (Behrens et al., 1987). Hence, the unfavorable deactivation of adamplatin(IV) by GSH does not seem to be a strong determinant of cytotoxic effects of this new drug. It is noteworthy that MT-2 does not react with adamplatin(IV). Complicating factors in the reaction of MTs with platinum(IV) drugs is their reduction by the cysteine thiols leading to cystine formation either intra- or intermolecularly and the many cysteine thiols in metallothioneins that are buried in metal-thiolate clusters (Zhong et al., 1997).

In conclusion, the results of the present work suggest that deactivation of adamplatin(IV) by sulfur-containing compounds (such as glutathione or metallothioneins) is likely to play a less significant role in the mechanism of resistance of

tumor cells to adamplatin(IV) in contrast to the role of this factor in the effects of cisplatin. Moreover, the treatment of tumor cells with adamplatin(IV) does not result in DNA modifications that would be markedly different from those produced by cisplatin. Conversely, the effects of other pharmacological factors associated with antitumor effects of adamplatin(IV), such as enhanced accumulation of the drug in cells, strong inhibition of DNA polymerization by these adducts, enhanced persistence of the adducts due to their less efficient repair and DNA-protein cross-linking are different from the effects of these factors in the mechanism underlying activity of cisplatin. Hence, the differences between the effects of adamplatin(IV) and cisplatin observed in the present work may help understand the unique activity of adamplatin(IV).

The cytotoxicity is the result of many events, beginning with the cell accumulation and proceeding through detoxification by thiols, DNA modification, and cellular responses to the DNA damage. It is not a simple task to reveal all aspects of the mechanism underlying antitumor effects of platinum complexes, and it is apparently incorrect to attribute cytotoxicity to their single property. Further studies are therefore warranted to reveal a relative contribution of all potential factors contributing to the potency of adamplatin(IV) in various types of cancer cells. Nevertheless, full clinical testing will only answer the fundamental question whether adamplatin(IV) represents a breakthrough in the development of antitumor platinum drugs.

Acknowledgments

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