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Conformation and recognition of DNA modified by a new antitumor dinuclear Pt^{II} complex resistant to decomposition by sulfur nucleophiles

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Abstract

Reported herein is a detailed biochemical and molecular biophysics study of the molecular mechanism of action of antitumor dinuclear Pt^{II} complex $[\{PtCl(DACH)\}_2-\mu-Y]^{4+}$ [DACH = 1,2-diaminocyclohexane, Y = H₂N(CH₂)₆NH₂(CH₂)₂NH₂(CH₂)₆NH₂] (complex **1**). This new, long-chain bifunctional dinuclear Pt^{II} complex is resistant to metabolic decomposition by sulfur-containing nucleophiles. The results show that DNA adducts of **1** can largely escape repair and yet inhibit very effectively transcription so that they should persist longer than those of conventional cisplatin. Hence, they could trigger a number of downstream cellular effects different from those triggered in cancer cells by DNA adducts of cisplatin. This might lead to the therapeutic effects that could radically improve chemotherapy by platinum complexes. In addition, the findings of the present work make new insights into mechanisms associated with antitumor effects of dinuclear/trinuclear Pt^{II} complexes possible.

Keywords

Antitumor; Dinuclear platinum complex; DNA damage; DNA repair; RNA polymerase II; Glutathione

1. Introduction

The development of new platinum-centered complexes as antitumor DNA-interactive agents has traditionally been focused on mononuclear bidentate platinum complexes. In order to contribute to the design of new antitumor platinum drugs we and others have been systematically testing the hypothesis that there is a correlation between clinical efficacy of platinum compounds and their ability to induce a certain sort of damage or conformational change in target DNA or, in other words, that platinum drugs that bind to DNA in a manner

fundamentally different from that of conventional *cis*-diamminedichloridoplatinum(II) (cisplatin) and its analogues can exhibit altered biological properties including the spectrum and intensity of antitumor activity [1,2]. Recent work based on testing this working hypothesis has focused on polynuclear platinum agents, the trinuclear [$\{trans\text{-PtCl}(\text{NH}_3)_2\}_2(\mu\text{-trans-Pt}(\text{NH}_3)_2[\text{NH}_2(\text{CH}_2)_6\text{NH}_2]_2)\}^{4+}$ (1,0,1/t,t,t, BBR 3464), which has undergone phase II clinical trials, being the prototype of this class [3–5]. DNA adducts of polynuclear platinum complexes differ significantly in structure and type from those of mononuclear platinum complexes. Especially, long-range intra- and interstrand crosslinks are formed in DNA which affect DNA conformation, are recognized by specific proteins and repaired differently in comparison with the crosslinks of cisplatin and its mononuclear analogues [6–10].

The biological activity of polynuclear platinum complexes may be modulated by the geometry and number of leaving groups in the coordination sphere of platinum atoms as well as the nature of the linking the platinum centers. Interestingly, the antitumor activity of the polyamine-linked dinuclear complexes is in many respects comparable to that of BBR3464 [11]. In contrast with the mononuclear complexes, such as antitumor cisplatin and clinically ineffective transplatin, in the dinuclear case both geometries are antitumor active [11], although DNA adducts (crosslinks) as well as local conformational distortions on DNA and their recognition by cellular components are affected by geometry [6,9,12]. The dinuclear *cis* isomer [$\{cis\text{-PtCl}(\text{NH}_3)_2\}_2(\text{H}_2\text{N}-(\text{CH}_2)_6-\text{NH}_2)\}^{2+}$ (1,1/c,c) is kinetically more inert in its reactions with DNA and in double-stranded DNA produces more interstrand crosslinks than its *trans* counterpart [9].

Differences between the two geometric dinuclear isomers are also manifested in their reactions with sulfur nucleophiles. The polynuclear motif of BBR3464, and the general structure, such as [$\{trans\text{-PtCl}(\text{NH}_3)_2\}_2\text{-}\mu\text{-Y}\}^{n+}$ (Y = $\text{NH}_2(\text{CH}_2)_6\text{NH}_2$, spermidine, spermine, etc.), is susceptible to decomposition since substitution of the Pt–Cl bond by a *trans*-influencing S donor results in bridge cleavage [13]. Similarly, a newer member of the polynuclear antitumor Pt^{II} family, the dinuclear BBR3610 [$\{trans\text{-PtCl}(\text{NH}_3)_2\}_2\text{-}\mu\text{-}\{trans\text{-}(\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_6\text{NH}_2)\}(\text{NO}_3)_4$ (Fig. 1), which was designed to mimic the same distance between the two Pt–Cl units in trinuclear BBR3464 [14,15], is decomposed by the sulfur nucleophiles [16]. Hence, this metabolic effect is likely to be highly deactivating because the capacity to form long-range crosslinks is lost. In contrast, the *cis* geometry as in [$\{cis\text{-PtCl}(\text{NH}_3)_2\}_2\text{-}\mu\text{-Y}\}^{n+}$ does not undergo decomposition of the dinuclear structure upon reaction with sulfur nucleophiles. In the case of thiols, novel thiolate-bridged macrocycles are formed, and with the thioether such as methionine, slow loss of NH_3 is eventually observed [17].

In an effort to design a polynuclear platinum compound that maintains the target (DNA) binding profile of the “parent” compound and is less susceptible to metabolic decomposition, the new, long-chain bifunctional dinuclear platinum complex structurally cognate with BBR3610 – [$\{\text{PtCl}(\text{DACH})\}_2\text{-}\mu\text{-Y}\}^{4+}$ [DACH = 1,2-diaminocyclohexane, Y = $\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_6\text{NH}_2$ (spermine-like chain)] (complex **1**, Fig. 1) – was synthesized [18]. Importantly, preliminary results showed that there was no labilization of the polyamine spermine-like linker groups of **1** in the presence of sulfur-containing species at physiological pH, which was attributed to the chelate effect of the DACH ring [18]. Complex **1** should, besides enhanced stability to metabolic deactivation, also exhibit other main features of potent di- or trinuclear platinum compounds. Besides reactions with sulfur-containing compounds, other important pharmacological features dictating a drug’s efficacy include cell accumulation, platinum–DNA binding and processing of platinated DNA by proteins (enzymes) and DNA repair. Thus, the primary objective in the present study was to understand more deeply the molecular mechanism of action of **1**. Here, we describe

experiments revealing (i) the DNA binding mode of **1** in cell-free media; (ii) the effects of DNA adducts of **1** on processivity of RNA polymerase II (the enzyme RNA pol II has been a major focus of the experiments designed to investigate cellular responses to DNA damage by platinum drugs); (iii) repair of DNA adducts of **1** (since the integrity of this process in human cells is a key indicator of the sensitivity of a tumor to platinum-based therapy); (iv) reactivity of **1** with glutathione (GSH), which has been chosen for investigation in this study because of its role as a determinant of cellular sensitivity to a wide variety of drugs and cytotoxic agents; and (v) cellular accumulation of **1**. For comparative purposes most of the experiments performed with **1** were also performed with BBR3610, to further study its mechanism of action as the complex exhibits a remarkable efficacy against glioma and colon cancer cells in culture and animal models [14,15].

2. Materials and methods

2.1. Chemicals

Cisplatin and transplatin were obtained from Sigma (Prague, Czech Republic) (purity was $\geq 99.9\%$ based on elemental and ICP trace analysis). Complex **1** and BBR3610 were prepared as described previously [18,19]. [PtCl(dien)]Cl (dien = diethylenetriamine) was a kind gift of prof. Giovanni Natile (University of Bari). Purity of **1**, BBR3610 and [PtCl(dien)]Cl was higher than 95% as established by combustion analysis carried out with a Hewlett Packard 185 C, H, and N analyzer. The stock solutions of platinum compounds were prepared at the concentration of 5×10^{-4} M in 10 mM NaClO₄ and stored at 4 °C in the dark. Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 20 000 kDa) was prepared and characterized as described previously [20,21]. Plasmids pSP73 [2464 base pairs (bp)], pUC19 (2 686 bp), and pBR322 (4361 bp) were isolated according to standard procedures. The Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the 3' → 5' proofreading domain), restriction endonucleases EcoRI, SspI, and XbaI, Circum Vent™ Thermal Cycle Sequencing Kit with Vent(exo⁻) DNA polymerase, and plasmid DNA pCMV-GLuc (5764 bp) were purchased from New England Biolabs (Beverly, MA). HeLaScribe® Nuclear Extract *in vitro* Transcription system kit was from Promega (Mannheim, Germany). GSH was from Sigma (Prague, Czech Republic). Deoxyribonucleotide triphosphates were from Roche Diagnostics, GmbH (Mannheim, Germany). Agarose was from FMC BioProducts (Rockland, ME). Acrylamide, bis(acrylamide), ethidium bromide (EtBr), and urea were from Merck KgaA (Darmstadt, Germany). Proteinase K and ATP were from Boehringer (Mannheim, Germany). Radioactive products were from Amersham (Arlington Heights, IL, USA). A cell-free extract (CFE) was prepared from the repair-proficient HeLa S3 cell line as described [22].

2.2. Platination reactions in cell-free media

If not stated otherwise, CT or plasmid DNAs were incubated with the platinum complex in 10 mM NaClO₄ at 37 °C in the dark. After 24 h, the samples were exhaustively dialyzed against the medium required for subsequent biochemical or biophysical analysis. An aliquot of these samples was used to determine the value of r_b (the number of molecules of the platinum complex bound per nucleotide residue) by flameless atomic absorption spectrometry (FAAS), or by differential pulse polarography (DPP) [23].

2.3. Sequence preference of DNA adducts

The primer extension footprinting assay was used to evaluate the sequence selectivity of DNA modification by **1** or BBR3610 in comparison to cisplatin and transplatin. The fragment of pSP73 DNA linearized by HpaI (2464 bp) was incubated with platinum complexes in 10 mM NaClO₄ for 24 h at 37 °C to obtain $r_b = 0.005$ or 0.01. The excess of drug was removed by ethanol precipitation. Circum Vent™ Thermal Cycle Sequencing Kit

with Vent(exo⁻) DNA polymerase was used along with the protocol for thermal cycle DNA sequencing with 5' end-labeled 20-mer SP6 primer recommended by the manufacturer with small modifications [24]. The synthesis products were separated by electrophoresis on a denaturing PAA gel [6% polyacrylamide (PAA)/8 M urea]; sequence ladders were obtained in parallel using untreated control DNA fragment.

2.4. DNA repair synthesis by human cell extract

Repair DNA synthesis of CFEs was assayed using pUC19 plasmid. Each reaction of 50 μ L contained 600 ng of nonmodified pBR322 and 600 ng of nonmodified or platinated pUC19, 2 mM ATP, 30 mM KCl, 0.05 mg/mL creatine phosphokinase (rabbit muscle), 20 mM each dGTP, dATP, and dTTP, 8 mM dCTP, 74 kBq of [α -³²P]dCTP 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 of bovine serum albumin/mL, and 20 μ g of CFE from the HeLa S3 cells. Reactions were incubated for 3 h at 30 °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 20 min. The products were extracted with one volume of 1:1 phenol/chloroform. The DNA was precipitated from the aqueous layer by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. After 30 min of incubation at -20 °C and centrifugation at 12 000 $\times$ g for 30 min at 4 °C, the pellet was washed with 0.2 mL of 80% ethanol and dried in a vacuum centrifuge. DNA was finally linearized before electrophoresis on a 1% agarose gel. Resulting gel was stained by EtBr. The experiments were made in quadruplicate.

2.5. Transcription by RNA polymerase II in vitro

In vitro transcription reaction was performed using HeLaScribe[®]Nuclear Extract *in vitro* Transcription system kit. The system contains all necessary components for *in vitro* transcription from a CMV promoter of plasmid DNA pCMV-GLuc. Plasmid pCMV-GLuc linearized by XbaI was incubated with platinum complexes in 10 mM NaClO₄ for 24 h at 37 °C to obtain $r_b = 8 \times 10^{-4}$ to 6.4×10^{-3} . After modification, DNA was precipitated by ethanol to remove incidental free platinum so that the samples contained no free platinum complex. *In vitro* transcription assay was performed using the HeLa nuclear extract supplied with the kit following the manufacturer's protocol with small modifications. Briefly, 100 ng of platinated or nonplatinated linearized pCMV-GLuc DNA was incubated in the 1 $\times$ transcription buffer supplemented by 4 mM MgCl₂, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rGTP, 16 μ M UTP, 10 μ Ci [α -³²P]UTP (3000 Ci/mmol), 20 U RNase inhibitor and nuclear extract (8 U) in a final reaction volume of 25 μ L at 30 °C for 60 min. The reaction was terminated by the addition of 175 μ L HeLa Extract Stop Solution followed by extraction with phenol and chloroform. The transcripts were precipitated by ethanol and the pellet was washed, dried and resuspended in a loading buffer containing 90% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue. The samples were electrophoresed through 6% denaturing PAA gel. The gels were subsequently visualized and the radioactivities associated with bands corresponding to full length runoff transcription products were quantitated.

2.6. Reactions with glutathione

Reactions of GSH with **1**, BBR3610, or cisplatin were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH exactly as described in the previous work [25]; the absorbance at 260 nm reflects the presence of platinum-sulfur and disulfide bonds. The platinum compounds (from aged stock solutions equilibrated in 10 mM NaClO₄) were mixed with GSH at 37 °C in the medium of 10 mM NaClO₄ plus 0.1 mM phosphate buffer, pH 7.0, in the dark and in nitrogen atmosphere. Reactions were initiated by mixing the platinum complex with the buffer followed by

immediate addition of GSH. The kinetic data were fitted by non-linear regression (GraphPad Prism) to one-phase or two-phase exponential association. The decision as to which fit was more appropriate for each dependence was made by comparing the fits of two equations by using an *F* test (GraphPad Prism).

2.7. Cellular platinum accumulation

Cellular uptake of **1**, BBR3610, and cisplatin was measured in A2780 and A2780cisR cells (sensitive and resistant to cisplatin, respectively). The cells were seeded in 10 cm tissue culture dishes (30 000 per cm²). After overnight incubation, A2780 and A2780cisR cells were treated for 4, 15 or 24 h with the platinum compound at the equimolar concentrations of 2.8 or 18.6 μ M, respectively (these concentrations correspond to the IC₅₀ values obtained for A2780 and A2780cisR cells treated with cisplatin for 72 h). The attached cells were washed twice with phosphate buffered saline (4 °C), the pellet stored at –80 °C. Afterward, the pellets were digested with 12 M HCl and platinum content determined by FAAS. All experiments were made in quadruplicate.

2.8. Other physical methods

Absorption spectra were measured with a Beckman 7400 DU spectrophotometer and quartz cells with a thermoelectrically controlled cell holder and a path length of 1 cm. Purification of oligonucleotides with the aid of HPLC was carried out on a Waters HPLC system consisting of Waters 262 pump, Waters 2487 UV detector, and Waters 600S controller with MonoQ HR 5/50 GL column. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For FAAS analyses, DNA was precipitated with ethanol and dissolved in HCl (0.1 M) whereas cells were mineralized using Milestone MLS 1200 Mega high performance microwave digestion unit with MLS Mega 240 terminal and EM 45/A Exhaust module. DPP was performed with an EG&G Princeton Applied Research Corporation Model 384B Polarographic Analyzer. The gels were visualized by using a BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivity associated with bands was quantitated with the AIDA image analyzer software (Raytest, Germany).

3. Results

3.1. DNA modification in a cell-free medium

3.1.1. DNA binding—Alterations in the target DNA induced by platinum drugs are important factors involved in the mechanisms of antitumor effects of these metallodrugs. In the present study, we first investigated the reaction products of **1** with CT DNA in cell-free media and compared these binding properties with those of its BBR3610 congener and cisplatin. The first experiments were aimed at quantifying the binding of **1** to mammalian DNA. Solutions of double-helical CT DNA at a concentration of 0.032 mg/mL were incubated with **1** at the value of r_1 of 0.05 in 10 mM NaClO₄ at 37 °C (r_1 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 DPP for platinum not bound to DNA. No changes in the pH of the reaction mixture containing DNA and platinum compounds were measured within 48 h after mixing DNA with the platinum complex. The amount of the platinum compound bound to DNA increased with time and the time at which the binding of **1** and BBR3610 reached 50% ($t_{50\%}$) was 87.4 ± 0.8 and 78.0 ± 0.8 min, respectively (Table 1). Thus, complexes **1** and BBR3610 were quantitatively bound after 24 h.

The binding experiments of the present work indicate that the modification reactions resulted in the irreversible coordination of **1** to polymeric double-helical DNA, which also

facilitates sample analysis. Hence, it is possible to prepare easily and precisely the samples of DNA modified by the platinum complex at a preselected value of r_b . The samples of DNA modified by **1** or BBR3610 and cisplatin and analyzed further by biophysical or biochemical methods were prepared in 10 mM NaClO₄ at 37 °C. If not stated otherwise, after 24 h of the reaction of DNA with the complex, the samples were precipitated in ethanol, dissolved in the appropriate medium and the r_b value in an aliquot of this sample checked by FAAS. In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

3.1.2. Replication mapping of platinum–DNA adducts—This procedure involved the extension by VentR(exo[−]) DNA polymerase at the 3′-end of the primer up to the metal adduct on the template strand of pSP73 DNA. The products of the synthesis were then examined on DNA sequencing gels, and the sequence specificity of the platinum adduct formation was determined to the exact base pair. *In vitro* DNA synthesis on DNA templates containing the adducts of **1** generated a population of DNA fragments, indicating that the adducts of this complex effectively terminated DNA synthesis (Fig. 2A, lanes 1 and 2). Sequence analysis of the termination sites produced by adducts of **1** shows that the major stop sites were similar to those produced by BBR3610 (Fig. 2A, lanes 3 and 4) and were mainly at guanine residues. While DNA synthesis on the template containing the adducts of cisplatin was terminated preferentially at the GG sites (Fig. 2A, lane 5) as expected, the termination due to the adducts of **1** or BBR3610 was less regular and rather similar to that due to the adducts of transplatin (Fig. 2A, lane 6), i.e. some stop sites were also at the C bases.

3.1.3. Interstrand crosslinking—Bifunctional platinum compounds, which coordinate base residues in DNA, form various types of interstrand and intrastrand crosslinks. Therefore, we have quantitated the interstrand crosslinking efficiency of **1** in pSP73 plasmid (2464 bp). This plasmid DNA was linearized by EcoRI (EcoRI cuts only once within pSP73 plasmid) and globally modified by **1** or BBR3610. The samples were analyzed for interstrand crosslinks by agarose gel electrophoresis under denaturing conditions [26]. Upon electrophoresis, 3′-end labeled strands of linearized pSP73 plasmid containing no interstrand crosslinks migrate as a 2464-base single strand, whereas the interstrand crosslinked strands migrate more slowly as a higher molecular mass species (Fig. S1A). The experiments were carried out with DNA samples that were modified by the platinum complex at various r_b values. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of noncrosslinked or crosslinked DNA under each condition. Interstrand crosslinking efficiency of **1** ($23 \pm 4\%$) was very similar to that of BBR3610 ($26 \pm 5\%$) (Table 1), implying that interstrand crosslinks represent a significant portion of the adducts formed in DNA by **1**.

3.1.4. Ethidium bromide fluorescence—The fluorescent probe EtBr can be used to distinguish between perturbations induced in DNA by monofunctional and bifunctional adducts of platinum compounds [8,27]. Binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by formation of the bifunctional adducts of a series of platinum complexes including cisplatin and transplatin, which results in a loss of fluorescence intensity.

Double-helical CT DNA was first modified by platinum complexes (**1**, BBR3610, cisplatin, or monofunctional [PtCl(dien)]Cl) for 24 h. The levels of the modification corresponded to the values of r_b in the range between 0 and 0.1. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Fig. 3). The decrease caused by the adducts of the dinuclear platinum complexes **1** and BBR3610, was similar and markedly more pronounced than that induced by the DNA adducts of cisplatin at equivalent r_b values.

Modification of DNA by the monofunctional platinum complex [PtCl(dien)]Cl resulted in only a slight decrease of EtBr fluorescence intensity as compared with the control DNA–EtBr complex [8,27]. The structures of DNA adducts of bifunctional dinuclear platinum compounds arise from two monofunctional substitutions on the polynucleotide. Comparison with [PtCl(dien)]Cl suggests that the conformational distortion induced in DNA by these adducts is much more delocalized and extends over considerably more base pairs around the platination sites than in the case of the adducts of mononuclear complexes such as cisplatin and its analogues. Thus, these results are consistent with the formation of long-range crosslinks by dinuclear **1** and BBR3610.

3.1.5. Unwinding of negatively supercoiled DNA—Electrophoresis in native agarose gel was used to determine the unwinding induced in negatively supercoiled pSP73 plasmid by monitoring the degree of supercoiling [28] (Fig. S2). A compound that unwinds the DNA duplex reduces the number of supercoils in closed circular DNA so that their number decreases. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible to observe and quantify the mean value of unwinding per one adduct. Fig. S4 shows electrophoresis gels from the experiment in which variable amounts of **1** or BBR3610 have been bound to a mixture of relaxed and negatively supercoiled pSP73 DNA. The mean unwinding angle is given by $\Phi = -18\sigma/r_b(c)$, where σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and nicked forms co-migrate [28]. Under the present experimental conditions, σ was calculated to be -0.036 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13^\circ$ was assumed. Using this approach, the DNA unwinding angle of $13 \pm 2^\circ$ or $14 \pm 2^\circ$ was determined for **1** and BBR3610, respectively (Table 1).

3.2. DNA repair synthesis by human cell extract

To investigate processing of adducts of complex **1** by DNA damage–recognition proteins, we used *in vitro* systems to study repair of platinum lesions by CFE and assay to examine catalytic activity of RNA polymerase II (RNA pol II) in human CFE to study the ability of the adducts of **1** to inhibit transcription by RNA pol II.

DNA repair efficiency in pUC19 plasmid (2686 bp) globally modified by **1**, BBR3610 or cisplatin at $r_b = 0.03$ was tested using CFE of repair-proficient HeLa cells. Repair activity was monitored by measurement of the amount of incorporated radiolabelled nucleotide. The incorporation of radioactive material was corrected for the relative DNA content in each band. As illustrated in Fig. 4, damage-induced DNA repair synthesis detected in the plasmid modified by **1** or BBR3610 was approximately 60% of that found for the cisplatin at the same level of modification.

3.3. RNA polymerase II transcription in human cell extracts

The effect of **1** on transcription activity of human RNA pol II was tested using the commercially available HeLaScribe® Nuclear Extract *in vitro* Transcription system kit. Using an analogous procedure as described earlier [29], the RNA pol II transcription template pCMV-Gluc either modified by **1** or cisplatin or nonmodified was incubated with HeLa nuclear extract supplied with this kit. This extract can support accurate transcription initiation by RNA pol II and exhibits both basal and regulated patterns of RNA transcription [30]. This nuclear extract also is the source for a variety of transcription factors, DNA binding proteins and the enzymatic machinery involved in process of RNA synthesis. Specific transcription from the CMV promoter results in a runoff transcript 688 nucleotides in length. The generated full length transcripts can be subsequently detected by gel electrophoresis. As seen in Fig. 5A, in the absence of platination, a high level of full length transcript was observed. In contrast, a significant decrease in the amount of full length

transcript was observed as a result of increasing template modification by both platinum complexes tested in this experiment. The relative amount of full length transcript generated from each reaction was quantitated and plotted as a function of the level of the template platination (r_b) (Fig. 5B). Under the conditions employed, RNA pol II transcription was highly sensitive to even very low levels of platinum damage on the template DNA, the damage by **1** being considerably more effective in inhibiting RNA pol II transcription than that by cisplatin.

To investigate the possibility that RNA pol II catalytic activity was inhibited as a consequence of hijacking factors essential for RNA pol II initiation by DNA adducts of **1** [29,31], the following competition experiments were performed. RNA pol II transcription of undamaged template pCMV-Gluc was examined in the presence of increasing levels of a second, exogenous pUC19 plasmid containing multiple lesions caused by either **1** or cisplatin. As shown in Fig. 6, the initial addition of control, undamaged exogenous plasmid resulted in an overall increase in the amount of transcript generated by pCMV-Gluc which only slightly increased upon the further addition of unplatinated plasmid. RNA pol II transcription of pCMV-Gluc template was significantly reduced by the addition of increasing amounts of cisplatin modified exogenous plasmid. In contrast, pronouncedly lower inhibition effect was seen if the transcription assay was performed in the presence of exogenous plasmid containing adducts of **1** (Fig. 6).

3.4. Reactions with reduced glutathione

Pt^{II} 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 [32]. Therefore, interest in the interactions of platinum antitumor drugs with sulfur-containing molecules of biological significance has recently increased markedly. An example of endogenous thiols to which platinum complexes bind when they are administered intravenously or after they enter the cell is GSH.

In the present work, we investigated reactions of GSH with **1**, BBR3610 and cisplatin using UV absorption spectrophotometry monitoring absorbance at 260 nm [25] (Fig. S3). The platinum complexes at a concentration of 33 μ M were mixed with 15 mM GSH (this concentration of GSH is physiologically relevant [33]) at 37 °C. The $t_{1/2}$ (half-times of the reactions, which mainly result in the formation of platinum–sulfur bonds) were 84, 135, and 109 min for reactions of **1**, BBR3610, and cisplatin with GSH, respectively (one-phase exponential association). Thus, **1** reacted with GSH markedly faster than BBR3610.

The absorbance at 260 nm reflects the presence of platinum–sulfur and disulfide bonds [25]. Interestingly, the absorbance at 260 nm determined for the reaction of GSH with **1** was approximately 2-fold and 1.3-fold lower than that determined for the reaction of GSH with BBR3610 and cisplatin, respectively (Fig. S2, Table 1).

3.5. Cellular accumulation

A factor contributing to platinum drug cytotoxicity is cellular accumulation. To examine accumulation of **1**, the cellular levels of this compound were measured after a 24 h exposure of the A2780 or A2780cisR cells to the drugs. The uptake of **1** by cisplatin sensitive A2780 cells was lower than that of BBR3610, but markedly greater than that of cisplatin (Fig. S4A and Table 1). On the other hand, the uptake of **1** by cisplatin-resistant A2780cisR cells was similar to that of BBR3610 and again markedly higher than that of cisplatin (Fig. S4B and

Table 1). These results are consistent with the previous findings demonstrating that dinuclear complexes carrying polyamine linkers are able to enter the cells more easily and faster than their mononuclear counterparts. Thus, the results show that there is no clear correlation between uptake and cytotoxicity of **1**, BBR3610 and cisplatin within the tumor cells sensitive and resistant to cisplatin. Nevertheless, the results do demonstrate the enhanced uptake of **1** and BBR3610 so that at least part of the increased cytotoxicity of **1** and BBR3610 over cisplatin in cisplatin-resistant cells seems to be attributable to an increased intracellular accumulation of **1** or BBR3610.

4. Discussion

The important target of platinum antitumor drugs leading to cell death is genomic DNA [34], although targets other than DNA may be also important for these agents [35–37]. Hence, it is generally accepted that one of important features of mechanism of action of cisplatin and its analogues is their binding to nuclear DNA and subsequent blockage of transcription while eluding repair [34,38]. This fact was impetus for the study described in this article.

It has been suggested [13,16] that metabolic decomposition of antitumor polynuclear platinum complexes by sulfur nucleophiles resulting in linker cleavage and loss of the di/trinuclear structure due to displacement of the Pt–Cl bond of di/trinuclear agents may explain why clinical tests of this class of antitumor metallodrugs, while giving some positive responses, did not reflect the high antitumor activity in preclinical experiments [5,11]. The search for a more suitable polynuclear platinum compound has led to the synthesis of the dinuclear complex **1** based on 1,2-DACH as carrier ligand and containing a spermine-like linker [18]. While dinuclear polyamine-linked drugs have been shown to behave toward DNA in a way generally similar to trinuclear BBR3464 [39] the DNA binding with this specific “6-2-6” linker has not been studied. Further the use of DACH as carrier group produces enhanced resistance to decomposition by sulfur nucleophiles, reasonably attributed to the chelate effect of the DACH ring. The remarkable biological effects of polynuclear platinum complexes have been attributed to their unique DNA binding mode. Thus, it is of interest to answer the question whether the new complexes **1** and BBR3610 exhibit a DNA binding profile similar to other antitumor di/trinuclear platinum agents, and whether there are significant differences between **1** and BBR3610, based on the former's resistance to metabolic decomposition by sulfur nucleophiles.

The major feature of DNA binding mode of BBR3464 and other antitumor dinuclear platinum compounds is that these compounds form long-range intra- and interstrand crosslinks; these crosslinks distort DNA conformation, but are not recognized by DNA binding proteins which readily bind to DNA modified by cisplatin [7]. The results of the present work confirm that also complexes **1** and BBR3610 form on DNA long-range crosslinks (Fig. 3). Similarly as other polynuclear Pt^{II} complexes, they form relatively high amount of interstrand crosslinks (23% and 26%, respectively, Table 1, Fig. S1). Trinuclear BBR3464 forms in DNA approximately 20% interstrand crosslinks which have been shown to be most likely lesions responsible for antitumor effects of this trinuclear agent [7]. Moreover, adducts of **1** and BBR3610 unwind DNA by approx. 13° on average (Table 1, Fig. S2), which is again a value very similar to that found for DNA unwinding by adducts of BBR3464 (14° [8]).

Thus, in broad terms, we have demonstrated that the DNA binding mode of **1** and BBR3610, including sequence preference, type of the major adducts, and resulting conformational alterations, is not very different from that of other antitumor di/trinuclear platinum drugs (Table 1). In addition, we have demonstrated that the major adducts of **1** and BBR3610 are

removed from DNA by DNA repair systems present in CFEs with a markedly lower efficiency than the adducts of cisplatin (Fig. 4 and Table 1). Hence, this result lends support to the view that these adducts persist longer on DNA than the adducts of cisplatin, which may potentiate antitumor effects and contribute to a lower resistance of the tumor cells to this metallodrug.

Studies focused on early phases of molecular mechanisms underlying antitumor effects of platinum antitumor drugs also involve investigations of cellular responses to DNA damage by these metallodrugs [40–42]. In the last 5 years particular attention has been paid to the role of inhibition of transcription elongation by RNA pol II by cisplatin adducts in mechanisms of their biological effects [38,43–46]. This important component of the mechanism underlying antitumor effects of platinum drugs has not been hitherto investigated in the case of di/trinuclear platinum compounds. We demonstrate in the present work for the first time (Figs. 5 and 6) that RNA pol II transcription is highly sensitive to even very low levels of modification on the template DNA by **1**. Thus, already very low levels of DNA modification by **1** may initiate transcription-coupled subpathways leading to apoptosis or necrosis [47].

Importantly, adducts of the dinuclear complex **1** are markedly more effective in inhibiting RNA pol II transcription than those of conventional cisplatin (Fig. 5). This different reduction in transcript production may result from different levels of platination and/or conformational distortions at the promoter, leading to promoter inactivation. The inhibition effects, however, were observed at the levels of platination well below that expected to induce such damage [$r_b = 8 \times 10^{-4}$, i.e. only ~9 adducts per molecule of template DNA (5764 bp) on average] so that this alternative is unlikely. An alternative possibility is that platinum lesions represent a sterical blockage of RNA pol II inhibiting the prolongation of RNA transcript or that factors essential for RNA pol II initiation may also bind Pt–DNA adducts which do not represent their natural binding sites. Therefore, in the presence of sufficient numbers of Pt–DNA adducts, these factors may become limiting, resulting in an inhibition of transcription initiation due to the factors being sequestered to platinated sites in DNA.

We examined in the present work whether some elements of transcription complex might be hijacked by DNA adducts of complex **1** more or less than by those of cisplatin using a competition assay [29] (Fig. 6). The initial addition of control, unplatinated exogenous pUC19 plasmid resulted in an overall increase in the amount of transcript generated by pCMV-Gluc substrate which increased only slightly upon the further addition of unplatinated exogenous plasmid. This increase in transcription efficiency has been already reported [29] and was attributed to an increase in macromolecular crowding induced by the presence higher amounts of DNA [29,48]. We observed in the present work (Fig. 6), in accord with the previously published results [29], that RNA pol II transcription of pCMV-Gluc template was markedly reduced by the addition of increasing amounts of cisplatin modified exogenous plasmid (Fig. 6). Thus, these results are consistent with the view and support the ‘hijacking’ hypothesis raised earlier for mononuclear Pt^{II} drugs [29,34,49,50] that RNA pol II transcription of template modified by cisplatin was inhibited in a substantial extent due to titrating some elements of transcription complex from their normal binding sites and in this way interfering with transcription.

On the other hand, a markedly lower inhibition effect was seen if the transcription assay was performed in the presence of exogenous plasmid modified by complex **1** (Fig. 6). Thus, the stronger inhibition of transcription by DNA adducts of **1** appears to be due to pronouncedly more extensive sterical blockade of RNA pol II inhibiting the prolongation of RNA

transcript than because of sequestering elements of transcription complex to platinated sites in DNA.

The observation of the present work that transcription elongation by RNA pol II was inhibited by titrating some elements of transcription complex from their normal binding sites much more by DNA adducts of cisplatin than by those of dinuclear complex **1** (Fig. 6) deserves further discussion. Many transcription factors are HMG-domain proteins (HMG = high mobility group). These proteins have been shown to recognize and bind with a strong affinity to structural motifs in DNA that involve directional rigid bends of the longitudinal axis of this double-helical nucleic acid. Major DNA adducts of cisplatin (intrastrand crosslinks between neighboring purine residues which represent ~90% of all DNA adducts of cisplatin [51]) produce in DNA a stable directional curvature (~40° toward major groove of DNA) [34]. Therefore, this bending apparently represents an important structural motif responsible for a high affinity of many transcription factors to DNA modified by cisplatin. On the other hand, major DNA adducts of antitumor di/trinuclear Pt^{II} compounds (long-range intra- and interstrand crosslinks) rather produce in DNA a flexible and only small nondirectional bend (~10°) so that these crosslinks are not recognized by HMG-domain proteins [6,7,10]. Hence, DNA adducts of **1** and other antitumor di/trinuclear Pt^{II} compounds may lack, in contrast to DNA adducts of cisplatin, a high-affinity structural motif which would attract transcription factors.

Given the similarities in DNA binding between **1** and BBR3610 it was of interest to examine other pharmacological factors which might differentiate the biological activity of the two compounds. The present study also reveals that the cellular uptake of both compounds is considerably greater than that of cisplatin (Table 1 and Fig. S4). The cellular accumulation was also enhanced in A2780cisR cells resistant to cisplatin over sensitive A2780 cells (Table 1), suggesting a mechanism for enhancement of tumor cell selectivity toward cisplatin-resistant cells. Thus, the results confirm a general property of charged di/trinuclear Pt^{II} complexes, i.e. the enhanced accumulation [11,52,53], so that at least part of the cytotoxicity of the new complex **1** seems to be attributable to an increased intracellular platinum concentration.

The results of the present work also demonstrate that **1** reacts with GSH with a higher rate compared with cisplatin (Table 1 and Fig. S3). Both clinical and preclinical studies have shown that cells with an elevated level of GSH (>10 mM) may be resistant to cisplatin and its analogues [54,55]. Thus, although it has been shown that reaction of **1** with GSH does not result in linker cleavage and loss of its dinuclear structure, further studies are warranted to determine whether deactivation by GSH is a strongly unfavorable determinant of cytotoxic effects of **1**. This may be important because the significant difference between **1** and BBR3610 is in the consequences of reaction with GSH. BBR3610 undergoes decomposition of its dinuclear structure upon reaction with sulfur nucleophiles [16]. Despite this unfavorable attribute, BBR3610 has been shown to exhibit a remarkable efficacy against glioma and colon cancer cells in culture and animal models [14,15], which provides a framework for investigation of mechanism of its action. This drug also reacts with GSH with a higher rate compared with cisplatin (Table 1 and Fig. S3), but in this case loss of linker is expected [15,56] which may unfavorably affect its toxicity. Further studies are warranted to dissect what part of the cytotoxicity of BBR3610 is attributable to its increased intracellular accumulation and is unfavorably affected by its faster reaction with GSH.

Results describing reactions of **1**, BBR3610 and cisplatin with GSH showed that the absorbance at 260 nm determined for the reaction of GSH with **1** was approximately 2-fold or 1.3-fold lower than that determined for the reaction of GSH with BBR3610 or cisplatin, respectively (Fig. S3, Table 1). Complex **1** based on 1,2-DACH as carrier ligand shows

enhanced resistance to decomposition in the presence of sulfur nucleophiles so that the products afforded by the reaction of GSH with **1** contained only one GSH molecule coordinated per each Pt atom in **1** [18]. The absorbance at 260 nm reflects the presence of platinum–sulfur and disulfide bonds [25,57], which can be interpreted to mean that under conditions of our experiments, i.e. in the presence of a surplus of GSH, the major products of the reaction of this tripeptide with BBR3610 contained 2-fold amount of GSH per Pt atom than those afforded by the reaction of GSH with complex **1**. This interpretation is in excellent agreement with previous findings [13] showing that the degradation of the trans geometry in di-/trinuclear compounds in presence of the surplus of GSH results in two mononuclear bisulfur Pt^{II} species. *cis*-oriented compounds containing NH₃ nonleaving groups initially remain intact upon reaction with GSH; the reactions are slower than those with the trans isomers, but eventually the NH₃ group trans to the sulfur atom is lost [17]. Hence, this slow liberation of NH₃ from *cis*-[PtCl₂(NH₃)₂] may result in some amount of mononuclear bisulfur Pt^{II} species at the expense of those containing only one sulfur-containing compound thereby increasing the mean number of sulfur-containing molecules bound per Pt^{II} atom in products afforded by the reaction of GSH with cisplatin. Hence, the 1.3-fold higher absorption at 260 nm observed for the reaction of GSH with cisplatin (Fig. S3, Table 1) compared with the reaction of GSH with **1** (whose resistance to decomposition by sulfur nucleophiles is attributed to the chelate effect of the DACH ring [18]), may imply that ca. 30% products afforded by the reaction of GSH initially containing only one sulfur-containing molecule were slowly transformed to bisulfur Pt^{II} species.

In conclusion, DNA adducts of dinuclear complex **1** not only block transcription markedly more efficiently than those of cisplatin, but also repair of adducts of **1** by the mammalian repair systems is markedly more reduced relative to repair of cisplatin (Fig. 4). Since DNA adducts of **1** can largely escape repair and yet inhibit very effectively transcription, they should persist longer than those of cisplatin. Hence, they could trigger a number of downstream cellular effects different from those triggered in cancer cells by DNA adducts of cisplatin. This might lead to the therapeutic effects that could radically improve chemotherapy by platinum complexes or could be at least an alternative to chemotherapy by platinum complexes already used in the clinic. Together, the findings of the present work help understand differential pharmacological effects of **1** or BBR3610 and conventional mononuclear cisplatin and thereby make new insights into mechanisms associated with antitumor effects of dinuclear/trinuclear platinum complexes possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BBR3610	$[\{trans\text{-PtCl}(\text{NH}_3)_2\}_2\text{-}\mu\text{-}\{trans\text{-(H}_2\text{N}(\text{CH}_2)_6\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_6\text{NH}_2)\}](\text{NO}_3)_4$
bp	base pair
CFE	cell-free extract
cisplatin	<i>cis</i> -diamminedichloridoplatinum(II)
complex 1	$[\{\text{PtCl}(\text{DACH})\}_2\text{-}\mu\text{-H}_2\text{N}(\text{CH}_2)_6\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_6\text{NH}_2]^{4+}$
CT	calf thymus
DACH	1,2-diaminocyclohexane

dien	diethylenetriamine
DPP	differential pulse polarography
EtBr	ethidiumbromide
FAAS	flameless atomic absorption spectrometry
GSH	glutathione
PAA	polyacrylamide
r_b	the number of molecules of the platinum complex bound per nucleotide residue
r_i	the molar ratio of free platinum complex to nucleotides at the onset of incubation with DNA
RNA pol II	RNA polymerase II

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Fig. 1.
Structures of the platinum complexes.

Fig. 2.

Replication mapping of platinum–DNA adducts. (A) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by VentR DNA polymerase on the pSP73 plasmid DNA linearized by HpaI restriction enzyme and subsequently modified by platinum complexes. The gel contained the linear amplification products of control, nonplatinated DNA and DNA treated with **1**, BBR3610, cisplatin or transplatin. Lanes: 1 and 2, DNA modified by **1** at $r_b = 0.005$ and 0.01 , respectively; 3 and 4, DNA modified by BBR3610 at $r_b = 0.005$ and 0.01 , respectively, C, G, T, A, chain-terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand); 5,6, DNA modified cisplatin or transplatin, respectively at $r_b = 0.01$;

7, unmodified template. The numbers correspond to the nucleotide sequence numbering of (B). (B) Schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of platinum complexes. The arrow indicates the direction of the synthesis. ●, major stop signals from (A), lane 5; ○, major stop signals from part A, lane 6; ▲, major stop signals from (A), lanes 1–4. The numbering of the nucleotides in this scheme corresponds to the numbering of the nucleotides in the pSP73 nucleotide sequence map.

Fig. 3. Ethidium bromide fluorescence. Dependences of the EtBr fluorescence on r_b for CT DNA modified by various platinum complexes in 10 mM NaClO₄ at 37 °C for 24 h. (■), complex 1; (▽), BBR3610; (Δ), cisplatin; (○) [PtCl(dien)]Cl.

Fig. 4.

In vitro DNA 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 or modified at $r_b = 0.03$ by cisplatin, complex **1**, and BBR3610. (A) Results of a typical experiment. Top panel, a photograph of the EtBr stained gel; bottom panel, autoradiogram of the gel showing incorporation of [α - ^{32}P]dCMP. Lanes: 1, nonmodified pBR322 plus pUC19 plasmids; 2, nonmodified pBR322 plus pUC19 modified by cisplatin; 3, nonmodified pBR322 plus pUC19 modified by **1**; 4, nonmodified pBR322 plus pUC19 modified by BBR3610. (B) Incorporation of dCMP into nonmodified or platinated pUC19 plasmid. 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%. Bars indicate SEM.

Fig. 5. Inhibition of RNA polymerase II transcription by DNA adducts of complex **1** or cisplatin. (A) Autoradiogram of the 8% PAA/8 M urea denaturing gel. Lanes: M, 100 bp DNA marker; 1, control, nonplatinated pCMV-Gluc substrate; 2, 3, and 4, pCMV-Gluc substrate modified by cisplatin at $r_b = 8 \times 10^{-4}$, 1.6×10^{-3} and 3.2×10^{-3} , respectively; 5, 6 and 7, pCMV-Gluc substrate modified by complex **1** at $r_b = 8 \times 10^{-4}$, 1.6×10^{-3} and 3.2×10^{-3} , respectively; 8, nonmodified substrate, no extract added. (B) Quantitative assessment. The relative transcription was assessed as follows: the amount of full length transcript at each r_b was quantitated (in % of total radioactivity in the lane) and calculated as the percentage of that generated by the control, nondamaged template. Data represent results of two independent experiments and are expressed as mean percentage $\pm$ SEM. For other details, see the text.

Fig. 6.

Inhibition of RNA polymerase II transcription by the addition of exogenously platinated DNA. Plasmid pUC19 unmodified or modified by cisplatin or complex **1** at $r_b = 0.05$ was used as exogenous DNA substrates. (A) Autoradiogram of the 8% PAA/8 Murea denaturing gel. Lanes: 1, transcription experiment was performed in the absence of exogenous plasmid (control); 2, 3 and 4, transcription experiment was performed in the presence of 30, 60, and 120 ng of exogenous unmodified plasmid, respectively; 5, 6 and 7, transcription experiment was performed in the presence of 30, 60, and 120 ng of exogenous plasmid modified by complex **1**, respectively; 8, 9 and 10, transcription experiment was performed in the presence of 30, 60, and 120 ng of exogenous plasmid modified by cisplatin, respectively. (B) The amount of full length transcript in each lane expressed as a mean fraction ($\pm$ SEM) of that generated in the absence of exogenously added DNA (white bar). The black bars represent transcription in the presence of undamaged DNA, the gray bars transcription in the presence of cisplatin modified DNA and the light gray bars transcription in the presence of DNA modified by complex **1**. For other details, see the text.

Table 1Summary of the effects of complex **1**, BBR3610 and cisplatin observed in this work.

	1	BBR3610	Cisplatin
DNA binding ($t_{50\%}$) (min) ^a	87.4 ± 0.8	78.0 ± 0.8	120 ^b
Preferential DNA binding sites ^c	G, GG, C	G, GG, C	GG, AG
% interstrand crosslinks/adduct ^a	23 ± 4	26 ± 5	6 ^d
Reduction of EtBr fluorescence	Strong	Strong	Medium
Plasmid DNA unwinding angle/adduct ^a	13 ± 2°	14 ± 2°	13° ^e
Inhibition of transcription by RNA pol II ^a	Strong	ND	Medium
DNA repair synthesis (%) ^a	55 ± 7	58 ± 3	100
Reaction with GSH ^a			
$t_{1/2}$ (min)	84	135	109
Absorbance at 260 nm after 200 min	0.17	0.35	0.22
Uptake (pmole Pt complex/10 ⁶ cells) after 24 h treatment			
By A2780 cells ^f	37 ± 3	71 ± 14	20 ± 11
By A2780cisR cells ^g	203 ± 41	213 ± 5	84 ± 15

^a See the text for details.^b Ref. [58].^c Determined by replication mapping.^d Ref. [26].^e Ref. [28].^f Cells were treated with the Pt complex at the concentration of 2.8 μM.^g Cells were treated with the Pt complex at the concentration of 18.6 μM.