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DNA interactions of new antitumor platinum complexes with *trans* geometry activated by a 2-metylbutylamine or *sec*-butylamine ligand

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Abstract

The global modification of mammalian and plasmid DNAs by novel platinum compounds, *trans*-[$PtCl_2(NH_3)$(Am)], where Am = 2-methylbutylamine or *sec*-butylamine was investigated in cell-free media using various biochemical and biophysical methods. These modifications were analyzed in the context of the activity of these new compounds in several tumor cell lines including those resistant to antitumor *cis*-diamminedichloroplatinum(II) (cisplatin). The results showed that the replacement of one amine group by 2-methylbutylamine or *sec*-butylamine ligand in clinically ineffective *trans*-diamminedichloroplatinum(II) (transplatin) resulted in a radical enhancement of its activity in tumor cell lines so that they are more cytotoxic than cisplatin and exhibited significant antitumor activity including activity in cisplatin-resistant tumor cells. Importantly, this replacement also markedly altered DNA binding mode of transplatin and reduced the efficiency of repair systems to remove the adducts of the new analogues from DNA. The results support the view that one strategy to activate *trans* geometry in bifunctional platinum(II) compounds including circumvention of resistance to cisplatin may consist in a chemical modification of the ineffective transplatin which results in an increased efficiency to form DNA interstrand cross-links.

Keywords: Cisplatin; Transplatin; Antitumor drugs; DNA; Cross-link; Repair

1. Introduction

Cisplatin is a potent anticancer drug for the treatment of testicular and other germ-cell tumors [1], but its clinical use is limited by the diminished activity against a number of cancers, the acquired resistance developed by many tumors and severe side effects. Therefore, the search continues for an improved platinum antitumor agent and in this search the clinical inactivity of transplatin was considered up to recently a paradigm for the classical structure—pharmacological activity relationships of platinum drugs [2]. However, to this end several new analogues of transplatin which exhibit a different spectrum of cytostatic activity including activity in tumor cells resistant to cisplatin have been identified (for reviews see [3–5]). Examples of these antitumor *trans* platinum complexes are the analogues of transplatin in which one ammine group was replaced by the ligands, such as thiazole, piperidine, piperazine, 4-picoline, cyclohexylamine [3,5–7]; and the analogues containing iminoether groups of general formula *trans*-[$PtCl_2$(*E*-iminoether)$_2$] (iminoether=HN=C(OCH_3)CH_3) [3,5–7].

The analogues of transplatin with asymmetric aliphatic ligands, such as *trans*-[$PtCl_2$(Am)(isopropylamine)] where

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Abbreviations: bp, base pair; CD, circular dichroism; CFE, cell-free extract; cisplatin, *cis*-diamminedichloroplatinum(II); CL, cross-link; CT, calf thymus; DMS, dimethyl sulfate; DPP, differential pulse polarography; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectrophotometry; FPLC, fast protein liquid chromatography; HMG, high-mobility-group; IC_{50}, the concentration of the compound that afforded 50% cell killing; PAA, polyacrylamide; [Pt(dien)Cl]Cl, chlorodiethylenetriamineplatinum(II) chloride; r_b, the number of molecules of the platinum compound bound per nucleotide residue; r_i, the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; t_m, melting temperature; *trans*-metbut, *trans*-[$PtCl_2(NH_3)$(2-methylbutylamine)]; transplatin, *trans*-diamminedichloroplatinum(II); *trans*-secbut, *trans*-[$PtCl_2(NH_3)$(*sec*-butylamine)].

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Am = butylamine, dimethylamine, isopropylamine or propylamine represent another new class of antitumor platinum compounds [3,5–7]. These drugs exhibit a cytotoxic activity in cisplatin-sensitive cells comparable to cisplatin and considerably higher in several cisplatin-resistant tumor cells [8]. The results obtained suggest that circumvention of cisplatin-resistance might be related with the ability of these drugs to induce apoptosis [4,9,10].

It is generally accepted that DNA is the major pharmacological target of antitumor platinum complexes and that the formation of kinetically stable adducts of these compounds on DNA and their processing in tumor cells underlies the mechanism of antitumor effects of platinum drugs [11]. The ability to circumvent cisplatin resistance is also thought to be related to DNA binding properties of the platinum complexes and particularly to their ability to form lesions in DNA that are different from those formed by cisplatin [5,12,13]. Interestingly, the results obtained with the complex in which both ammine groups were replaced by dimethylamine and isopropylamine form on DNA more interstrand CLs than cisplatin or transplatin both on naked DNA in cell-free media and on DNA isolated from the cells treated with the platinum complex [4,14]. These results imply a new mechanism of action for this class of antitumor platinum compounds. The presence of the asymmetric aliphatic amines may result in altered hydrogen bonding and steric effects affecting the kinetics of DNA binding, the structures and/or stability of the adducts formed and resulting local conformational alterations in DNA.

Thus, in order to attain the ability to rationally design novel antitumor platinum drugs capable of circumventing inherent or acquired resistance to cisplatin, it is necessary to understand how the DNA binding properties of antitumor platinum complexes that are not cross-resistant with cisplatin differ from the DNA binding properties of cisplatin and transplatin. In this work we describe the DNA binding properties of *trans*-[$PtCl_2(NH_3)(Am)$], where Am = 2-methylbutylamine or *sec*-butylamine, see Fig. 1 for their structures and compare these binding properties with activity of the new compounds in several tumor cell lines including those resistant to cisplatin. These new *trans* compounds have been designed and synthesized with the aim to further improve knowledge on structure–pharmacological relationship of this class of platinum compounds. In contrast to antitumor *trans* compounds of this class synthesized and tested up to now (in which both NH_3 groups were replaced by an aliphatic ligand), the new *trans* complexes investigated in the present work still contain one NH_3 group enhancing their solubility in water solutions. Hence, this modification may result not only in alterations in the mechanism of their biological activity, but may be also important from the viewpoint of potential administration of these drugs in the clinic.

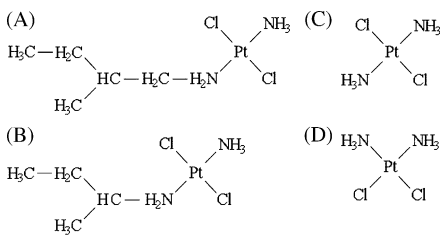

Fig. 1. Structures of platinum complexes used in this work.

2. Materials and methods

2.1. Starting materials

The *trans*-metbut and *trans*-secbut (Fig. 1A and B) were prepared by the methods described in detail previously [15]. Cisplatin, transplatin (Fig. 1C and D), glycogen and DMS were obtained from Sigma. [PtCl(dien)]Cl was kindly provided by G. Natile. The stock solutions of the platinum complexes at the concentration of 5×10^{-4} M in 10 mM $NaClO_4$ were prepared in the dark at 25°. CT DNA (42% G + C, mean molecular mass ca. 20,000 kDa) was prepared and characterized as described previously [16,17]. Plasmids pSP73KB (2455 bp), pSP73 (2464 bp), pUC19 (2686 bp) and pBR322 (4363 bp) were isolated according to standard procedures. Expression and purification of recombinant rat full-length HMGB1 protein were carried out as described [18,19]. The synthetic oligodeoxyribonucleotides were synthesized and purified as described previously [20]. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Klenow fragment of DNA polymerase I was from Boehringer-Mannheim Biochemica. A CFE was prepared from the HeLa S3 cell line as described [21,22]. This extract was kindly provided by J.T. Reardon and A. Sancar from the University of North Carolina. Acrylamide, agarose, bis(acrylamide), EtBr, urea, thiourea and NaCN were from Merck KgaA. Creatine phosphokinase and creatine phosphate were from ICN Biomedicals Inc. The radioactive products were from Amersham.

2.2. Platination reactions

CT or plasmid DNAs were incubated with the platinum complex in 10 mM $NaClO_4$ at 37° in the dark. After 48 hr, the samples of plasmid DNA were precipitated by ethanol and redissolved in the medium required for subsequent biochemical or biophysical analysis whereas the samples of CT DNA were exhaustively dialyzed against such a medium. An aliquot of these samples was used to determine the value of r_b by FAAS or DPP [23].

The single-stranded 22-mer oligodeoxyribonucleotides (pyrimidine-rich strands containing central CGCGC or TGTGT sequence) were reacted in stoichiometric amounts with transplatin and its analogues tested in the present work. The platinated oligonucleotides were repurified by

ion-exchange FPLC. It was verified by platinum FAAS and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA [24–26] that one platinum molecule was coordinated to two guanine residues at their N7 position. Other details have been described previously [20,26,27].

2.3. Unwinding of negatively supercoiled DNA

Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay [28]. The unwinding angle Φ, induced per Pt-DNA adduct was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of pSP73 plasmid were incubated with platinum compounds for 48 hr, precipitated by ethanol and redissolved in TAE buffer (0.04 M Tris–acetate + 1 mM EDTA, pH 7.0). An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25° in the dark with TAE buffer with a voltage set at 30 V. The gels were then stained with EtBr, followed by photography on Polaroid 667 film with transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

2.4. DNA melting

The melting curves of CT DNAs were recorded by measuring the absorbance at 260 nm. The melting curves were recorded in a medium containing 10 mM or 0.2 M $NaClO_4$ with 1 mM Tris–HCl/0.1 mM EDTA, pH 7.4. The value of the t_m was determined as the temperature corresponding to the maximum on the first-derivative profile of the melting curves. The t_m values could be determined with an accuracy of ±0.5°.

2.5. Gel-mobility-shift assay

The labeled 107-bp DNA probe with blunt ends was prepared from the *Nde*I/*Eco*RI fragment of pSP73 plasmid in which its overhanging ends were filled in by Klenow fragment of DNA polymerase I. The 3′-end labeled probe was modified by cisplatin, *trans*-metbut or *trans*-sec at r_b values in the range of 0–0.04. These probes were titrated with the HMGB1 protein in the presence of 0.1 mg/mL sonicated calf thymus DNA in the buffer composed of 10 mM HEPES, pH 7.9, 10 mM $MgCl_2$, 150 mM NaCl, 0.2 mg/mL bovine serum albumin, 20% v/v glycerol, and 1 mM dithiothreitol. The electrophoresis was performed for 50 min at 4°.

2.6. Repair synthesis by human cell extracts

Repair DNA synthesis of CFEs was assayed using pUC19 and pBR322 plasmids. Each reaction of 50 μL 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 μM of each dGMP, dCTP and TTP, 8 mM dATP, 74 kBq of [α-^{32}P]dAMP in the buffer composed of 40 mM HEPES–KOH, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 22 mM creatine phosphate, 1.4 mg/mL bovine serum albumine and 150 μg of CFE. Reactions were incubated for 3 hr at 25° and terminated by adding EDTA to a final concentration of 20 mM, SDS to 0.6% and proteinase K to 250 μg/mL and then incubating for 30 min. The products were extracted with 1 volume 1:1 phenol:chloroform. The DNA was precipitated from the aqueous layer by the addition of 1/50 volume 5 M NaCl, 5 μg glycogen and 2.5 volumes ethanol. After 20 min of incubation on dry ice and centrifugation at 12,000 *g* for 30 min at 4°, the pellet was washed with 0.5 mL 70% ethanol and dried in a vacuum centrifuge. DNA was finally linearized before electrophoresis on a 1% agarose gel containing 0.3 μg/mL EtBr.

2.7. Transcription mapping of DNA adducts

Transcription of the (*Nde*I/*Hpa*I) restriction fragment of pSP73KB DNA with 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 [25,26]. Before the aliquots containing the transcripts were loaded on the PAA gel, the radioactivity associated with these samples was adjusted so that equal amounts of the radioactivity was loaded into each well.

2.8. Interstrand cross-link assay

Platinum complexes at varying concentrations were incubated for 24 hr with 500 ng of the *Nde*I/*Eco*RI fragment of pUC19 DNA (213 bp) 3′-end labeled by means of Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The platinated samples were precipitated by ethanol, the pellets were dissolved in 18 μL of 30 mM NaOH with 1 mM EDTA, 6.6% sucrose and 0.04% bromophenol blue, and samples were immediately analyzed for platinum content by FAAS and for DNA interstrand CLs in the same way as described in recent papers [26,29,30]. Briefly, the amount of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand CLs (%ICL/Pt, the number of interstrand CLs per adduct), was calculated as $\%ICL/Pt = XL/426r_b$ (*Nde*I/*Eco*RI fragment of pSP73KB plasmid contained 426 nucleotide residues). XL is the number of interstrand

CLs per one molecule of the linearized DNA duplex which was calculated assuming Poisson distribution of the interstrand CLs as $XL = -\ln A$, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

2.9. Rearrangement of intrastrand cross-links of trans compounds

The oligodeoxyribonucleotides containing a single, site-specific 1,3-GNG intrastrand CL of transplatin, *trans*-metbut or *trans*-secbut (top strands of the duplexes CGCGC and TGTGT, see Fig. 8 for their sequences) at 20 μM were allowed to anneal with the unplatinated complementary strands in 0.2 M $NaClO_4$/5 mM Tris–HCl, pH 7.5/0.1 mM EDTA at 20° for 30 min and then for 2 hr at 4°. The resulting duplexes at 2 μM (or higher) were subsequently incubated at 37°. At various time intervals, aliquots were withdrawn and analyzed by electrophoresis in denaturing 12% PAA/8 M urea gel. The bases involved in the interstrand CLs were determined by Maxam–Gilbert footprinting [26,31].

2.10. Other physical methods

Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out on a Unicam 939 AA spectrometer with a graphite furnace. For FAAS analysis, DNA was precipitated with ethanol and dissolved in 0.1 M HCl. DPP curves were recorded with the aid of an EG&C PARC Electrochemical Analyzer, Model 384B. FPLC purification was carried out on a Pharmacia Biotech FPLC System with MonoQ HR 5/5 column. CD spectra were recorded at 25° using a JASCO spectropolarimeter, Model J720. The gels were dried and visualized by using the FUJIFILM bioimaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software.

2.11. Cytotoxicity

Culture of pairs of cisplatin sensitive and resistant ovarian cancer cell lines (A2780/A2780cisR and CH1/CH1cisR) were described elsewhere [32]. Cell survival in the cultures treated with the platinum complexes was evaluated by the microculture tetrazolium assay [33] as described in our recent paper [34]. Briefly, the platinum compounds were added to 96 microwell plates containing the cell cultures at final concentrations in the range of 0–0.2 mM. After 24 hr, cell survival was evaluated by measuring absorbance at 520 nm. IC_{50} values were calculated from the plots of cell survival vs. compound concentration. Experiments were performed in quadruplicate. Other details can be found in the previously published papers [32–34].

3. Results

3.1. DNA binding

Solutions of double-helical CT DNA at a concentration of 0.04 mg/mL were incubated with the platinum complexes tested in the present work at the value of r_i of 0.01 in 10 mM $NaClO_4$ at 37°. At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by DPP 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. No changes in the pH of the reaction mixture containing DNA and platinum compounds were measured within 48 hr after mixing DNA with the platinum complex. The amount of the platinum compounds bound to DNA increased with time. In these binding reactions the times at which the binding reached 50% ($t_{50\%}$) were 120 and 150 min for *trans*-metbut and *trans*-secbut, respectively. The value of $t_{50\%}$ for the reaction of cisplatin or transplatin with DNA under comparable conditions was ~120 min. These results indicate that the rates of binding of *trans*-metbut and *trans*-secbut to natural double-helical DNA are comparable to those of cisplatin or transplatin.

In further experiments, CT DNA was incubated with the new analogues of transplatin at $r_i = 0.2$ and essentially the same $t_{50\%}$ values of the binding were observed as at $r_i = 0.01$. The binding of these new platinum compounds to CT DNA was also quantified in the other way. Aliquots of the reaction withdrawn at various time intervals were quickly cooled on an ice bath and then exhaustively dialyzed against 10 mM $NaClO_4$ at 4° to remove free (unbound) platinum compound. The content of platinum in these DNA samples was determined by FAAS. Results identical to those obtained using the DPP assay were obtained. The binding experiments of the present work indicate that the modification reactions resulted in the irreversible coordination of the new analogues of transplatin 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 new platinum compounds and analyzed further by biophysical or biochemical methods were prepared in 10 mM $NaClO_4$ at 37°. If not stated otherwise, after 24 hr of the reaction of DNA with the complex the samples were precipitated in ethanol, dissolved in the medium necessary for a particular analysis and the r_b value in an aliquot of this sample was checked by FAAS. In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

3.2. Circular dichroism spectroscopy

CD spectral characteristics were compared for CT DNA in the absence and in the presence of the new analogues of

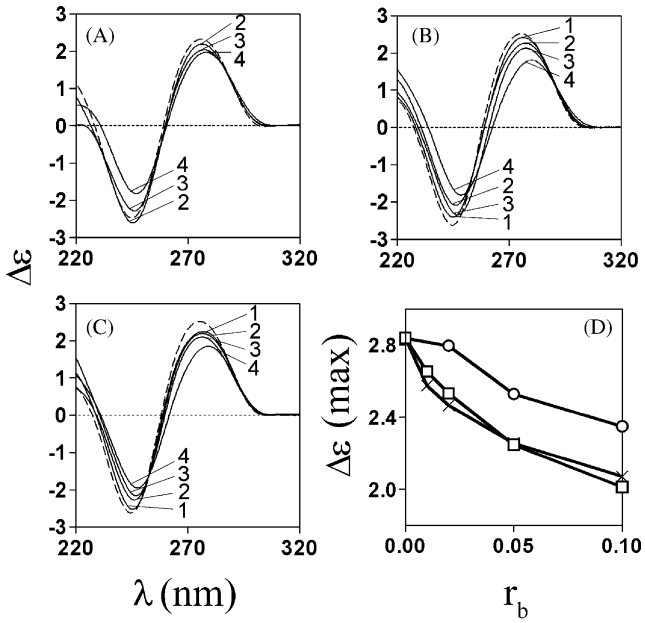

Fig. 2. CD spectroscopy of calf thymus DNA modified by transplatin and its analogues. CD spectra were recorded for DNA in 10 mM $NaClO_4$. CD spectra of DNA modified by transplatin (A), *trans*-metbut (B) and *trans*-secbut (C); curves: dashed lines—control (nonmodified) DNA; 1, $r_b = 0.01$; 2, $r_b = 0.02$; 3, $r_b = 0.05$; 4, $r_b = 0.1$. (D) Changes in the CD spectra of DNA at the maximum of the positive band (~275 nm): (○) transplatin, (□) *trans*-metbut, (×) *trans*-secbut.

transplatin tested in the present work at r_b values in the range 0–0.1 (Fig. 2). Upon binding of these compounds to CT DNA, the conservative CD spectrum normally found for DNA in canonical B-conformation considerably transforms at wavelengths below 300 nm. There was a significant decrease in the intensity of the positive band around 280 nm. Based on the analogy with the changes in the CD spectra of DNA modified by cisplatin and clinically ineffective transplatin [35], it might be suggested that the binding of both new transplatin analogues results in the conformational alterations in double-helical DNA of denaturational character similar to those induced in DNA by parent transplatin [35]. Thus, the CD results (Fig. 2) also suggest that the binding of *trans* analogues affects DNA conformation differently from antitumor cisplatin which induces in DNA nondenaturational alterations [35].

3.3. DNA unwinding

Electrophoresis in native agarose gel is used to determine the unwinding induced in negatively supercoiled pSP73 plasmid by monitoring the degree of supercoiling [28] (Fig. 3). 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.

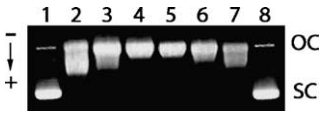

Fig. 3. Unwinding of supercoiled pSP73 plasmid DNA modified by *trans*-metbut. Lanes: 1, 8, control, nonmodified DNA; 2–7, $r_b = 0.06, 0.09, 0.11, 0.14, 0.16, 0.18$, respectively. The top bands correspond to the form of nicked plasmid (oc) and the bottom bands to closed negatively supercoiled plasmid (sc).

Figure 3 shows electrophoresis gel from the experiment in which variable amounts of *trans*-metbut have been bound to a mixture of relaxed and negatively supercoiled pUC19 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 comigrate [28]. Under the present experimental conditions, σ was calculated to be -0.055 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 angles of $8 \pm 1^\circ$ were determined. The same result was obtained for *trans*-secbut (not shown) so that the unwinding angle observed for both new analogues of transplatin are similar to those found for transplatin (9° [28]).

3.4. DNA melting

CT DNA was modified by transplatin or its new analogues tested in the present work to the value of r_b in the range 0–0.1 in 10 mM $NaClO_4$ at 37° for 24 hr. The samples were divided into two parts and in one part the salt concentration was further adjusted by addition of $NaClO_4$ to 0.2 M. Hence, the melting curves for DNA modified by the platinum compounds to the same level were measured in the two different media, at low and high salt concentrations. The effect on t_m is dependent on the salt concentration (Fig. 4). At both low and high salt concentrations (0.01 and 0.2 M, respectively) an increase of t_m is observed for the modifications by all *trans*-platinum compounds. Interestingly, the increase of t_m was markedly higher at the low salt concentration and the new analogues of transplatin enhanced t_m more than parent transplatin. Interestingly, cisplatin and its analogues thermally destabilize DNA [5].

3.5. Recognition by HMGB1 proteins

We examined the affinity of the full-length HMGB1 protein (HMG-domain proteins are known to mediate antitumor activity of cisplatin [5,36]) for DNA modified by new antitumor analogues of *trans*-metbut and *trans*-secbut. In these experiments, a 107-bp *Eco*RI/*Nde*I fragment of pSP73 plasmid (see Section 2), nonmodified or platinated by *trans*-metbut or *trans*-secbut and for comparative purposes also by cisplatin to r_b values in the range

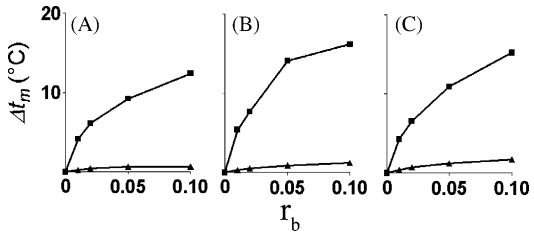

Fig. 4. Plots showing the dependence of Δt_m values on r_b for calf thymus DNA modified by transplatin and its analogues. (A) Transplatin; (B) *trans*-metbut; (C) *trans*-secbut. The melting curves were measured in 0.01 M (■) or 0.2 M (▲) $NaClO_4$ plus 1 mM Tris–HCl with 0.1 mM EDTA, pH 7.4. Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs. Data measured in triplicate varied on average ± 2% from their mean.

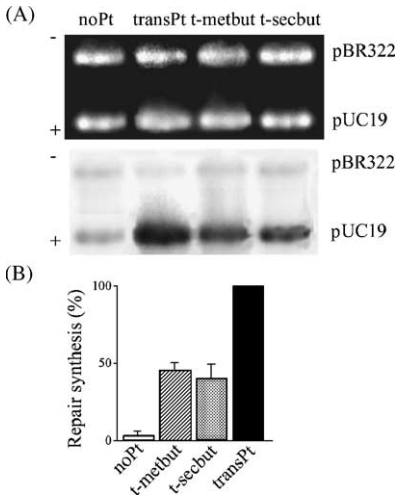

Fig. 5. *In vitro* repair synthesis assay of the extract prepared from the repair-proficient HeLa cell line. Repair synthesis used as substrates nonmodified pBR322 plasmid and pUC19 plasmid nonmodifed (lane noPt) or modified at $r_b = 0.05$ by transplatin, *trans*-metbut or *trans*-secbut (lanes transPt, t-metbut, t-secbut, respectively). (A) Results of a typical experiment. The top panel is a photograph of the EtBr stained gel and the bottom panel is the autoradiogram of the gel showing incorporation of [α-^{32}P]dAMP. (B) Incorporation of dAMP into nonmodified 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. Bars indicate SEM.

of 0.005–0.04, was used in electrophoretic mobility-shift assay with recombinant rat HMGB1 protein. Consistently with the previous reports [37] a shifted (more slowly migrating) band corresponding to the complex formed between 107-bp probe modified by cisplatin and the protein was observed (not shown), the intensity of which increased with growing protein concentration. This result confirmed affinity of HMGB1 protein for DNA modified by cisplatin [36,38]. No more slowly migrated species was detected if the protein was incubated with the 107-bp probe nonmodified or platinated by *trans*-metbut or *trans*-secbut under identical conditions. Thus, these results indicate that HMGB1 protein has no affinity to DNA modified by *trans*-metbut or *trans*-secbut similarly as to DNA modified by parent transplatin [37].

3.6. Repair synthesis by cell extracts

Figure 5A illustrates an experiment measuring DNA repair synthesis by a repair-proficient HeLa CFE in pUC19 plasmid platinated with *trans*-metbut, *trans*-secbut and transplatin at $r_b = 0.05$. Repair activity was monitored by measuring the amount of incorporated radiolabelled 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 platinated pUC19 plasmid. Approximately the same levels of damage-induced DNA repair synthesis were detected in the plasmid modified by *trans*-metbut and *trans*-secbut (Fig. 5A, lanes, *trans*-metbut and *trans*-secbut). In contrast, transplatin adducts induced a considerably higher level of repair synthesis which was approximately double that found for the adducts of its analogues (Fig. 5A, lane transPt and Fig. 5B).

3.7. In vitro transcription mapping of platinum adducts

There are several main methods that can be used to determine the preferential DNA-binding sites or sequence specificity of a DNA-binding agent [39]. In order to determine the sequence specificity of *trans*-metbut or *trans*-secbut we used in the present work a method which consists in RNA synthesis by T7 RNA polymerase *in vitro* in the same way as in several previous studies of the sequence specificity of various DNA-damaging agents including platinum drugs [25,26,40–45]. T7 RNA polymerase was chosen to initiate these investigations because it is well characterized, its promoter is clearly defined, and the purified enzyme is commercially available. RNA synthesis by various RNA polymerases including T7 RNA polymerase 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 [25,26,46]. Importantly, monofunctional DNA adducts of several platinum complexes including cisplatin and transplatin are unable to terminate RNA synthesis [25,26].

Cutting of pSP73KB DNA [25] by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 6B). This fragment contained T7 RNA polymerase promotor in the upper strand close to its 3′-end (Fig. 6B). The experiments were carried out using this linear DNA fragment, randomly modified by cisplatin, transplatin, transplatin analogues *trans*-metbut and *trans*-secbut or [Pt(dien)Cl]Cl at $r_b = 0.005$, for RNA synthesis by T7 RNA polymerase (Fig. 6A, lanes cisPt, transPt, *trans*-metbut, *trans*-secbut and dienPt, respectively). RNA synthesis on the template modified by the platinum complexes

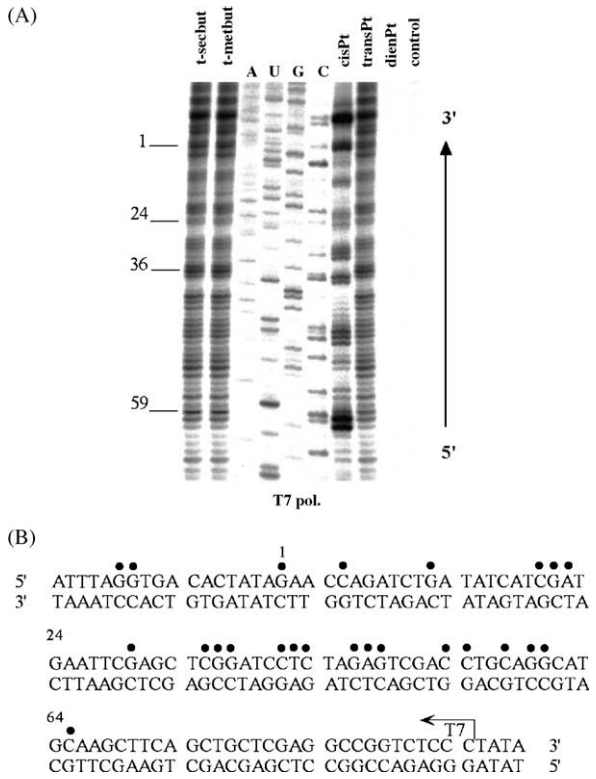

Fig. 6. Inhibition of RNA synthesis by T7 RNA polymerases on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid modified by platinum complexes. (A) Autoradiograms of 6% PAA/8 M urea sequencing gels showing inhibition of RNA synthesis by T7 RNA polymerase on the *Nde*I/*Hpa*I fragment containing adducts of platinum complexes. Lanes: control, unmodified template; t-metbut, t-secbut, cisPt, transPt and dienPt, the template modified by *trans*-metbut, *trans*-secbut, cisplatin, transplatin or [Pt(dien)Cl]Cl at $r_b = 0.005$, respectively; A, U, G and C, chain terminated marker RNAs. (B) Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by platinum complexes. The arrows indicate the start of the T7 RNA polymerase, which used as template the upper strand of *Nde*I/*Hpa*I fragment of pSP73KB DNA. (●), major stop signals (from A) for DNA modified by *trans*-metbut. The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

yielded fragments of defined sizes, which indicates that RNA synthesis on these templates except that on the template modified by [Pt(dien)Cl]Cl was prematurely terminated. The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of transplatin and its analogues were similar, appeared mainly at G and C sites and to a considerably less extent also at adenine (A) sites (Fig. 6B). Importantly, the sequence dependence of the inhibition of RNA synthesis by the adducts of transplatin and its analogues is considerably less regular than that by the adducts of cisplatin, indicating that the *trans* compounds form a greater variety of adducts with DNA and less regularly than does cisplatin. Consistently with the previous results [25,26,46] RNA synthesis on the template modified by monofunctional [Pt(dien)Cl]Cl was not prematurely terminated (Fig. 6A, lane dienPt).

3.8. Characterization of DNA adducts of transplatin analogues by thiourea

Cisplatin, transplatin and analogous bifunctional platinum compounds coordinate to DNA in a two-step process, forming first monofunctional adducts, preferentially at guanine residues, which subsequently close to bifunctional lesions [11,35,47–49]. Thiourea is used to labilize monofunctionally bound transplatin from DNA [50]. The displacement of transplatin is initiated by coordination of thiourea *trans* to the nucleobase. Because of the strong *trans* effect of sulfur, the nucleobase nitrogen–platinum bond is weakened and thus becomes susceptible to further substitution reactions. Consequently, transplatin in monofunctional DNA adducts is effectively removed, whereas bifunctional adducts of transplatin are resistant to thiourea treatment [50].

The experiments, aimed at the characterization of DNA adducts of transplatin and its analogues, were conducted employing thiourea as a probe for DNA monofunctional adducts formed by *trans*-platinum compounds [50]. Double-stranded DNA was incubated with transplatin or its analogues at a drug to nucleotide ratio of $r_i = 0.05$ in 10 mM $NaClO_4$ at 37°. The reaction was stopped after 24 hr by adjusting the NaCl concentration to 0.2 M and by immediate cooling to −20°. In parallel experiments, the reactions were stopped by addition of 10 mM thiourea solutions. These samples were incubated for 10 min at 37° and then quickly cooled to −20°. The samples were then exhaustively dialyzed against 0.2 M NaCl and subsequently against H_2O at 4°, and the platinum content was determined by FAAS.

Thiourea displaced 40 and 36% of transplatin and *trans*-metbut or *trans*-secbut from DNA, respectively. It can be concluded that after a reaction period of 24 hr, 60 and 64% of monofunctional adducts of transplatin [50,51] and *trans*-metbut or *trans*-secbut, respectively had evolved to bifunctional lesions.

3.9. Interstrand cross-linking

Bifunctional platinum compounds that covalently bind to DNA form various types of interstrand and intrastrand CLs. Considerable evidence suggests that the antitumor efficacy of bifunctional platinum compounds is the result of the formation of these lesions, but their relative efficacy remains unknown. Therefore, we have decided to quantitate the interstrand cross-linking efficiency of both analogues of transplatin tested in the present work in the 213-bp *Nde*I/*Eco*RI fragment of pUC19 plasmid modified by the platinum complexes. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions [26]. Upon electrophoresis, 3′-end labeled strands of linearized pSP73KB plasmid containing no interstrand CLs migrate as a 213-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (Fig. 7).

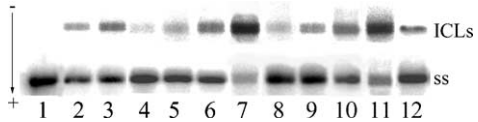

Fig. 7. The formation of the interstrand CLs by platinum complexes in linear *Nde*I/*Eco*RI of pSP73KB plasmid (213 bp). Autoradiogram of denaturing 1% agarose gels of linearized DNA which was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). The fragment was nonplatinated (control) (lane 1) or modified by transplatin at $r_b = 0.005$ or 0.01 (lanes 2 and 3, respectively); *trans*-metbut at $r_b = 0.0005$, 0.001, 0.002, 0.005 (lanes 4–7, respectively); *trans*-secbut at $r_b = 0.0005$, 0.001, 0.002, 0.005 (lanes 8–11, respectively) and by cisplatin at $r_b = 0.01$ (lane 12).

The experiments were carried out with DNA samples which were modified by the platinum complexes for 24 hr at various r_b values. The bands corresponding to more slowly migrating interstrand-cross-linked fragments were seen for r_b values as low as 5×10^{-4} (Fig. 7, lane 2). 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 (%ICL/Pt) 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. The DNA interstrand cross-linking efficiency of both new analogues of transplatin tested in the present work was almost independent of r_b (the frequency of the CLs was in the range 40–50%). Hence, the interstrand cross-linking efficiency found for the new analogues of transplatin was considerably higher than that found for parent transplatin (12%; [26]).

The samples of pUC19 DNA linearized by *Eco*RI and modified by the compounds tested in the present work at $r_b = 0.0005$ and 0.005 were also analyzed in 1% nondenaturing agarose gel (not shown). No new, more slowly migrating bands were observed, which indicates that no CLs between DNA strands belonging to different duplexes were formed.

3.10. Stability of the 1,3-GNG intrastrand cross-links of transplatin analogues

The 1,3-GNG intrastrand CL of transplatin (N = any nucleotide) is stable within single-stranded DNA under physiological conditions. Within double-helical DNA its stability is markedly reduced and is even notably smaller than that of the interstrand CLs (preferentially formed by this platinum compound between guanine and complementary cytosine residues). Consequently, the pairing of single-stranded DNA containing 1,3-GNG intrastrand CL of transplatin with their complementary DNA sequences results in rearrangement of these intrastrand adducts into interstrand CLs [52].

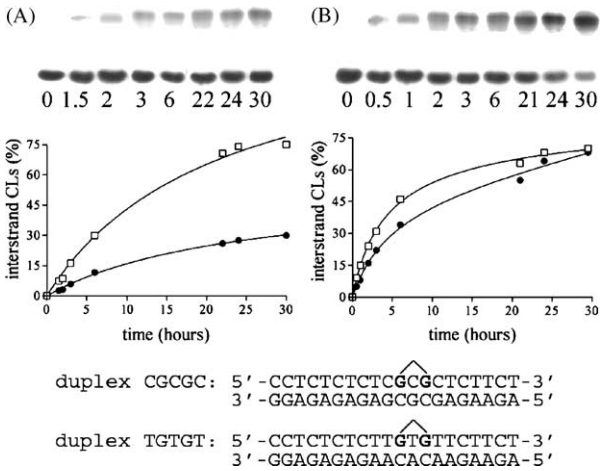

Fig. 8. Rearrangement of the 1,3-intrastrand CLs formed by transplatin (A) or *trans*-secbut (B) in the duplexes CGCGC (●) and TGTGT (□). The samples of the 2 μM duplexes were incubated at 37° in 0.2 M $NaClO_4$, 5 mM Tris–HCl buffer (pH 7.5) and 0.1 mM EDTA; at various time intervals, the aliquots were withdrawn and analyzed by electrophoresis in 12% PAA/8 M urea gel. Top parts of the panels: autoradiograms of the gels of the duplex CGCGC modified by transplatin or *trans*-sec radioactively labeled at the 5′-end of its top strand. Incubation times in hours are indicated under each lane. Lane 0 refer to the 5′-end labeled single-stranded top (platinated) strand. Bottom parts of the panels: plots of the percentages of the interstrand CLs vs. time. These percentages were calculated from the ratio of the radioactivity in each lane in the autoradiograms associated with the band corresponding to the lower bands to the sum of the radioactivities associated with both bands (multiplied by 100). For other details, see the text.

The stability of 1,3-GNG intrastrand CLs of transplatin and its analogues *trans*-metbut and *trans*-secbut was investigated using 20-mer oligodeoxyribonucleotides (the top strands of the duplexes CGCGC and TGTGT shown in Fig. 8 which were radioactively labeled at their 5′-ends) and platinated so that they contained single and central, site-specific 1,3-GCG or GTG intrastrand CL. The single-stranded oligonucleotides containing this CL or the corresponding duplexes were incubated in 0.2 M $NaClO_4$ at 37°. At various time intervals, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (Fig. 8).

The 1,3-GNG intrastrand adducts of both analogues of transplatin within the single-stranded oligonucleotide were inert over a long period of time (>5 days) (not shown). It was verified by DMS footprinting that no rearrangement of the 1,3-intrastrand CL occurred within this period. In contrast, this adduct formed by *trans*-metbut and *trans*-secbut in the central sequences CGCGC and TGTGT after pairing the platinated single-stranded oligonucleotides with their complementary strands was labile (shown in Fig. 8 for *trans*-secbut). As a function of time, the radioactivity associated with the band corresponding to the 1,3-intrastrand CL decreased with the concomitant increase of the radioactivity associated with the new, more slowly migrating species that migrated at the same rate as the 20-bp duplex containing a single, interstrand CL of transplatin (shown for the adduct formed by transplatin and

trans-secbut in the central sequences CGCGC and TGTGT in Fig. 8A and B, respectively). This result was interpreted to mean that the 1,3-intrastrand CL was transformed into an interstrand CL [52]. After 30 hr of the incubation of the duplex CGCGC and TGTGT containing the 1,3-intrastrand CL of *trans*-secbut, ~70% of the 1,3-intrastrand CLs were transformed into the interstrand CLs in both duplexes (Fig. 8B). Similar yields of these rearrangement reactions (not shown) were observed for the 1,3-intrastrand CLs of *trans*-metbut (not shown). Interestingly, after 30 hr of the incubation of the duplexes CGCGC and TGTGT containing the 1,3-intrastrand CL of transplatin only 26 and 70% of the 1,3-intrastrand CLs were transformed into the interstrand CLs (Fig. 8A). Hence, the yields of these rearrangement reactions involving the 1,3-intrastrand CLs of transplatin are similar or lower depending on the nucleotide sequence at the site of the intrastrand CL.

3.11. Cytotoxicity

The cytotoxic activity of the platinum compounds tested in the present work was determined against two pairs of cisplatin sensitive and resistant cancer cell lines (see Table 1) [34]. These pairs of cell lines were selected on the basis of encompassing the known major mechanisms underlying resistance to cisplatin: A2780cisR being resistant through a combination of decreased uptake, enhanced DNA repair/tolerance and elevated reduced glutathione levels and CH1cisR through enhanced DNA repair/tolerance. The compounds were incubated for 24 hr with the tumor cell lines and the cell survival in the culture treated with the platinum compounds was evaluated as described previously [33,34].

The IC_{50} values are shown in Table 1. The analogues of transplatin in which one ammine group was replaced by the 2-methylbutylamine or *sec*-butylamine ligand were radically more potent than parent transplatin in the lines both sensitive and resistant to cisplatin. Importantly, these analogues of transplatin were also more potent than cisplatin in the cell line encompassing some of the known major mechanisms of resistance to cisplatin, such as a combination of decreased uptake, enhanced DNA repair/tolerance, and elevated reduced glutathione levels. Thus, the replacement of one ammine ligand by the 2-methylbutylamine or *sec*-butylamine ligand markedly enhanced the activity of transplatin in cancer cell lines.

Table 1
IC_{50} (μM) mean values obtained for the platinum compounds tested in the present work

	A2780	A2780cisR	CH1	CH1cisR
Cisplatin	2.2	38 (17.3)	6.0	23.0 (3.8)
Transplatin	>200	>200	>200	>200
trans-metbut	1.7	9.3 (5.5)	4.2	15.1 (3.6)
trans-secbut	2.1	15.5 (7.4)	5.5	18.0 (3.3)

The numbers in parentheses are the resistance factor (IC_{50} resistant/IC_{50} sensitive).

The *trans*-metbut and *trans*-secbut are chiral drugs and the experiments described above were performed with their racemic mixtures. *R*- and *S*-enantiomers of these drugs were also prepared, but only in relatively small quantities due to difficulties associated with the separation of the two enantiomers. Therefore, the experiments described above including the determination of cytotoxic activity were also carried out with the *R*- and *S*-enantiomers although in a limited extent. Identical results for both enantiomers have been obtained as in the experiments in which the racemic mixtures were used.

4. Discussion

The results of the present work demonstrate that the replacement of one ammine group by the aliphatic ligand such as 2-methylbutylamine or *sec*-butylamine in the molecule of ineffective transplatin results in a radical enhancement of its activity in tumor cell lines so that they are more cytotoxic than cisplatin and exhibit significant antitumor activity including activity in cisplatin-resistant tumor cells (Table 1). Concomitantly, this replacement markedly alters the binding mode of transplatin to DNA (Table 2) which is generally accepted to be a major pharmacological target of antitumor platinum compounds [11]. Hence, the results of the present work further sustain the hypothesis that platinum drugs which bind to DNA in a fundamentally different manner to that of cisplatin may have altered pharmacological properties [5,6,53].

The results of the present work demonstrate that the analogues locally unwind DNA (Fig. 3, Table 2). The values of unwinding angles are affected by the nature of the ligands in the coordination sphere of platinum and the stereochemistry at the platinum center. A previous systematic work [28] revealed that the platinum(II) compounds fall into different classes according to their DNA binding modes. It has been shown that platinum(II) compounds with the smallest unwinding angles ($\leq 6^\circ$) are those that can bind DNA only monofunctionally {[PtCl(dien)]Cl or $[PtCl(NH_3)_3]Cl$}. Another group of platinum compounds is composed of those which bind to DNA in a bifunctional manner. The compounds which belong to this category unwind DNA by 10–13° and include cisplatin, transplatin and antitumor bifunctional polynuclear complexes like $[\{trans\text{-}PtCl(NH_3)_2\}_2(\mu\text{-}H_2N(CH_2)_nNH_2)]Cl_2$, $n = 2$–6. The observation that all new analogues of transplatin tested in the present work cannot be grouped with monofunctional platinum(II) compounds is readily understood in terms of adduct structures in which at least a part of the complexes are coordinated to DNA in a bifunctional manner.

The bifunctional adducts preferentially formed by transplatin in DNA are 1,3-GNG intrastrand CLs and interstrand

Table 2
Summary of DNA binding characteristics of *trans*-[$PtCl_2(NH_3)$(2-methylbutylamine)] (*trans*-metbut), *trans*-[$PtCl_2(NH_3)$(*sec*-butylamine)] (*trans*-secbut), cisplatin and transplatin

	trans-metbut	*trans*-secbut	Cisplatin	Transplatin
DNA binding ($t_{50\%}$)[a]	120 min	150 min	120 min[b]	120 min[b]
Percentage interstrand CLs/adduct[c]	40	50	6[d]	12[d]
Percentage monofunctional lesions/adduct[c]	36	36	~1	40
CD band at 278 nm[c]	Decrease	Decrease	Increase[e]	Decrease[e]
Unwinding angle/adduct[c]	8°	8°	13°[f]	9°[f]
Melting temperature (Δt_m)[c,g]				
Low ionic strength	14.1°	10.9°	(−2.5°)[h]	(9.2°)[h]
High ionic strength	0.9°	1.2°	(−4.1°)[h]	(0.6°)[h]
Stability of intrastrand CLs	Low	Low	High	Medium
DNA repair synthesis	Low	Low	Low	High

[a] The time at which the binding reached 50%.
[b] Bancroft *et al.* [49].
[c] DNA modified for 24 hr.
[d] Brabec and Leng [26].
[e] Brabec *et al.* [35].
[f] Keck and Lippard [28].
[g] Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs; the values correspond to $r_b = 0.05$.
[h] Zaludova *et al.* [57].

CLs formed between guanine and complementary cytosine residues [26,48,50]. It has been also demonstrated [52] that the intrastrand CLs formed by transplatin in several sequences of double-helical DNA are unstable and this property of transplatin was also related to its clinical inefficiency [54,55]. We have demonstrated in the present work (Fig. 8) that the replacement of one ammine ligand in transplatin by the 2-methylbutylamine or *sec*-butylamine ligand results in a low stability of the 1,3-intrastrand CL formed in the sequence in which transplatin forms a relatively stable intrastrand adduct. Thus, an overall stability of the 1,3-GNG intrastrand CLs formed by the analogues of transplatin containing the aliphatic amine ligand in double-helical DNA appears to be even lower than that observed for the intrastrand CLs of transplatin. Consistently with the low stability of the intrastrand CLs formed by *trans*-metbut or *trans*-secbut these adducts are formed in DNA randomly modified by these analogues with considerably lower frequency than the same adducts of transplatin and only represent the lesions formed in DNA by these new analogues with the lowest frequency (~24 or 14%, respectively, Table 2). Hence, the markedly higher activity of transplatin analogues containing the 2-methylbutylamine or *sec*-butylamine ligand in tumor cell lines is unlikely associated with the effects of their intrastrand CLs.

The clinical ineffectivity of transplatin has been proposed to be also associated with its reduced capability to form in double-helical DNA bifunctional adducts [55,56]. A considerably higher interstrand cross-linking efficiency of transplatin analogues containing the 2-methylbutylamine or *sec*-butylamine ligand in comparison with transplatin (Table 2) remains an important candidate for the factor responsible for their markedly higher activity in tumor cells. The CLs are formed in DNA by bifunctional platinum compounds in a two-step process [49]. In the first step, the monofunctional adducts are formed preferentially at guanine residues which subsequently close to the CLs. The unexpected high rate of interstrand CL formation by transplatin analogues containing the 2-methylbutylamine or *sec*-butylamine ligand relative to that determined for "classical" transplatin may imply that conformational changes in double-stranded DNA, induced by the aliphatic ligand in a monofunctional adduct, modulate the second binding step and facilitate formation of an interstrand CL, in contrast to the situation for parent transplatin. Further studies are required aimed at determining the sites involved in the interstrand CLs of these analogues and the conformational alterations induced in DNA by all types of adducts to identify more factors responsible for high interstrand cross-linking efficiency of this class of platinum compounds.

Melting of DNA modified by *trans*-metbut or *trans*-secbut (Fig. 4) also deserves discussion. An increase of t_m observed at low salt concentration (Table 2) can be interpreted to mean that under these conditions "stabilizing" effects such as also interstrand CLs and positive charges on platinum moieties dominate over the "destabilizing" effects of conformational alterations induced by the adducts of *trans*-platinum compounds [57]. That the adducts of new analogues of transplatin change conformation of DNA is supported by the results of DNA unwinding and CD experiments (*vide supra*). An interesting question is why high salt appears to result in lower overall stabilization due to the binding of the *trans* compounds. It is possible that the observed lower increase in t_m at high ionic strength is a consequence of the lower contribution of "stabilizing" effects of the adducts of transplatin, *trans*-metbut or *trans*-secbut. At high salt concentration the stabilizing effects are reduced since electrostatic effects

of the platinum compounds are apparently lowered with increasing concentration of Na^+ counter ions nevertheless the stabilizing factors still dominate over the destabilizing effects of conformational changes induced by its adducts. The melting of DNA modified by platinum compounds is a complex process which may involve other factors not considered in the present work. Thus, a more definitive evaluation of all effects of the replacement of ammine groups in transplatin by the aliphatic ligands on DNA melting remains to be determined.

Another important feature of the mechanism underlying antitumor effects of platinum compounds investigated in the present work is DNA repair synthesis induced by DNA adducts. A persistence of DNA adducts of platinum compounds may potentiate their antitumor effects in the cells sensitive to these compounds [5,38]. DNA repair synthesis was investigated in the present work using the CFE from human tumor cells and DNA substrates randomly modified by the platinum compounds (Fig. 5). Importantly, *trans*-metbut or *trans*-secbut adducts induced a considerably lower level of repair synthesis than the adducts of parent transplatin (Fig. 5B) suggesting a considerably less efficient removal from DNA and enhanced persistence of the adducts of the transplatin analogues in comparison with the adducts of the parent compound. As the analogues form considerably more interstrand CLs than transplatin also the latter observation supports an idea about an important role of these lesions formed by *trans*-metbut or *trans*-secbut in the mechanism underlying their antitumor effects. The interstrand CLs pose a special challenge to repair enzymes because they involve both strands of DNA and cannot be repaired using the information in the complementary strand for resynthesis. In general, DNA interstrand CLs could be even more effective lesions than other adducts in terminating DNA or RNA synthesis in tumor cells.

The most frequent lesions formed by *trans*-metbut or *trans*-secbut in double-helical DNA are apparently interstrand CLs (Table 2). This is a major feature of DNA binding mode of the new antitumor analogues of transplatin which makes it significantly different from DNA binding mode of cisplatin and transplatin. It cannot be excluded that the character of the interstrand CLs of *trans*-metbut or *trans*-secbut has structural features different from those of the interstrand CLs formed by cisplatin or transplatin. The different character of interstrand CLs of the new transplatin analogues tested in the present work might be at least partly responsible for their distinctly different biological effects in comparison with the effects of cisplatin or transplatin. Interestingly, DNA interstrand CLs have often been implicated in the cytotoxicity of platinum complexes [58–60].

In conclusion, the mechanism underlying antitumor activity of platinum compounds is a complex process involving a number of factors. Among these factors are also those that do not operate directly at the level of the DNA adducts, such as for instance those affecting the amount of platinum complex that can reach DNA [by influencing either the cell accumulation of the complexes or the levels of intracellular platinophiles that act as detoxification agents (metallothioneine and glutathione)]. Nevertheless, it is generally accepted that DNA is an important and major pharmacological target of platinum compounds [5,11,61]. The present work correlates DNA binding mode in a cell-free medium of the bifunctional analogues of transplatin containing one 2-methylbutylamine or *sec*-butylamine ligand and DNA repair synthesis with their activity in several tumor cell lines. At present it cannot be excluded that the cytotoxic effects may also be due to a cumulative effect of the structurally heterogeneous adducts produced by *trans*-platinum drugs. Data on conformation, recognition by DNA-binding proteins and repair of DNA interstrand CLs of *trans*-metbut or *trans*-secbut will provide more insights into which DNA adduct of these compounds is a more likely lesion responsible for antitumor effects of this class of platinum drugs.

The results offer a strong experimental support for the view that one strategy how to activate *trans* geometry in bifunctional platinum(II) compounds and to circumvent resistance to cisplatin consists in a chemical modification of the conventional transplatin which would result in their increased efficiency to form interstrand CLs. The present work also suggests that such a modification may be accomplished by the replacement of one ammine group by an aliphatic ammine ligand, such as 2-methylbutylamine or *sec*-butylamine ligand. Moreover, the analogues of transplatin apparently represent a novel class of platinum anticancer drugs acting by a different mechanism than cisplatin. A further understanding of how transplatin analogues with aliphatic amine ligands modify DNA and how these modifications are further processed in cells should provide a rational basis for the design of new platinum antitumor drugs and chemotherapeutic strategies.

Acknowledgments

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