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Toxicity in tumor cells, DNA binding mode, and resistance to decomposition by sulfur nucleophiles of new dinuclear bifunctional *trans*-Pt^{II} complexes containing long alkane linkers*

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Abstract: In an effort to design dinuclear Pt^{II} compounds that maintain the target (DNA) binding profile of the *trans*-oriented dinuclear bifunctional Pt^{II} complexes containing aliphatic linker chains but are less susceptible to metabolic decomposition, the new, long-chain dinuclear Pt^{II} complexes—$[\{trans\text{-PtCl(dien)}\}_2\text{-}\mu\text{-}(CH_2)_n]^{2+}$ (n = 7,10,12, dien = diethylenetriamine)—were synthesized. The toxicity of these metallodrugs was examined in ovarian tumor cell lines. The results showed that the activity of these complexes increased with growing length of the linker; the activity of complex containing the longest linker (n = 12) was comparable with that of *cis*-diamminedichloridoplatinum(II) (cisplatin). This observation correlated with the results of DNA binding studies performed in cell-free media. The results of these studies demonstrated that the growing length of the aliphatic bridge promoted more distorting conformational alterations induced in DNA. Attention was also paid to the reactivity of $\{[Pt(dien)Cl]_2\text{-alkane}\}$ compounds with glutathione (GSH). The results of these experiments support the thesis that the dinuclear structure of $\{[Pt(dien)Cl]_2\text{-alkane}\}$ complexes remains stable in the presence of S-containing compounds without undergoing chemical degradation as previously observed for some di/trinuclear bifunctional Pt^{II} complexes. This enhanced stability represents a favorable property which may contribute to reduce side effects and increase therapeutic efficacy of the dinuclear $\{[Pt(dien)Cl]_2\text{-alkane}\}$ compounds.

Keywords: anticancer activity; cytotoxicity; dinuclear platinum; DNA binding mode; glutathione; nucleic acids.

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INTRODUCTION

Polynuclear complexes represent a distinct class of Pt-based anticancer agents that contain two or more Pt centers linked by a chain of different length [1,2]. DNA binding, and the consequences for structure and function, is the mechanistic paradigm by which cytotoxic Pt complexes are believed to exert their antitumor activity [3–5]. Thus, antitumor polynuclear Pt complexes were designed as a result of systematically testing the hypothesis that Pt drugs that bind to DNA in a manner fundamentally different from that of the conventional mononuclear *cis*-diamminedichloridoplatinum(II) (cisplatin) and its analogues, can exhibit altered biological properties including the spectrum and intensity of antitumor activity [6,7]. The chemistry and biological activity of polynuclear Pt complexes may be modulated by the geometry and number of leaving groups in the coordination sphere as well as by the nature of the chain linking the Pt centers [1,2,8,9].

Initial studies on polynuclear Pt antitumor drugs focused on symmetric dinuclear bifunctional Pt^{II} complexes where the two Pt atoms have equivalent coordination spheres, represented by the general formula $[\{trans\text{-}PtCl(NH_3)_2\}_2\text{-}\mu\text{-}NH_2(CH_2)_nNH_2]^{2+}$, $n = 2–6$ (1,1/t,t-*n*, Fig. 1) [10]. The properties of these formally bifunctional complexes have been reported including their different spectrum of cytostatic activity and activity in tumor cells resistant to conventional cisplatin [11–14]. In addition, several papers have demonstrated unique DNA binding modes of these bifunctional dinuclear Pt^{II} compounds [10,15–19]. For instance, they preferentially form so called long-range cross-links (CLs) in which the platinated sites in DNA are separated by one or more base pairs. Moreover, in contrast to cisplatin, which forms on DNA ~90 % intrastrand CLs and only 6 % interstrand CLs [21], these dinuclear bifunctional Pt^{II} compounds form on DNA ~80 % interstrand CLs [20]. Also, as a consequence of the global (random) modification of natural, high-molecular-mass DNA by these dinuclear bifunctional Pt^{II} compounds, the conformation of this target biomacromolecule is altered in a way that is distinctly different from the modifications by cisplatin [10,17]. As might be expected, varying the length of the linker may affect the distance and spatial configuration of the reactive chloride groups in these dinuclear compounds, hence also their DNA binding mode and consequently biological effect. However, no systematic study of the effect of the length of the linker has been performed in particular for the complexes containing longer aliphatic linker chains such as in 1,1/t,t-$n > 6$.

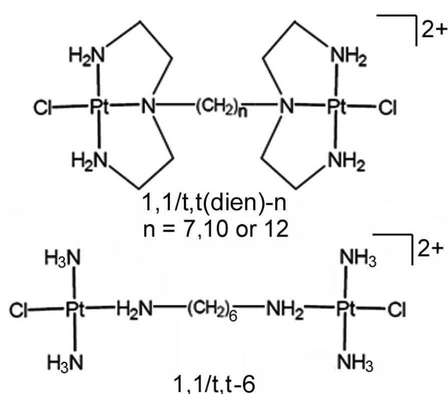

Fig. 1 Structures of the dinuclear bifunctional Pt^{II} complexes.

The disposition of leaving ligands (chlorides) in relation to the linkers (cis or trans) in polynuclear Pt^{II} compounds determines their susceptibility to metabolic decomposition by the S nucleophiles leading to inactivation. Decomposition occurs when the labile ligand is trans to the linker so substitution of the leaving ligand (e.g., chloride) by a *trans*-labilizing S donor results in breaking the bond between the

Pt center and the linker and liberation of the terminal amine of the alkane diamine linker [22,23]. Hence, this metabolic effect is likely to be highly deactivating and also results in loss of the capacity to form long-range CLs responsible for unique biological properties of dinuclear bifunctional Pt^{II} complexes.

In an effort to design dinuclear Pt^{II} compounds that maintain the target (DNA) binding profile of the "parent" compounds 1,1/t,t-*n* but are less susceptible to metabolic decomposition, the new, long-chain bifunctional dinuclear Pt^{II} complexes structurally cognate with the latter dinuclear complex—[{*trans*-PtCl(dien)}$_2$-μ-(CH$_2$)$_n$]$^{2+}$ (1,1/t,t(dien)-*n*, *n* = 7,10,12 and dien = diethylenetriamine, Fig. 1)—were synthesized. Importantly, preliminary results showed that there was no breakage of the linker groups of these new dinuclear Pt^{II} complexes in the presence of S-containing species at physiological pH. In addition, the preliminary results also revealed that DNA binding mode of the new dinuclear Pt^{II} compounds was dependent on the length of the linker. Therefore, we examined in the present work toxicity of 1,1/t,t(dien)-*n* (*n* = 7,10,12) in two tumor cell lines, cisplatin-sensitive A2780 and isogenic multifactorial-resistant A2780cisR, respectively. Attention was paid to the comparison of the effect of these longer aliphatic linkers on cytotoxicity and DNA-binding modes of these metallodrugs in a cell-free medium. Reactivity of 1,1/t,t(dien)-*n* compounds 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, was investigated as well.

RESULTS AND DISCUSSION

Cytotoxicity

The cytotoxic activity of 1,1/t,t(dien)-*n* (*n* = 7,10,12) and cisplatin was determined against human ovarian carcinoma cell lines A2780 (parent cisplatin-sensitive) and A2780cisR (acquired resistance to cisplatin) commonly used to test cytotoxic activity of cisplatin analogues and other antitumor metallodrugs. The cell lines were incubated for 72 h with the Pt compound, and the cell survival in the culture treated with the Pt compound was evaluated as described in the Supplementary Information. The three dinuclear Pt^{II} compounds showed activity in the tested concentration range, and their corresponding IC$_{50}$ values are reported in Table 1. In general, the activity of complexes 1,1/t,t(dien)-*n* (*n* = 7,10,12) increased with growing length of the linker in both cell lines. Interestingly, the activity of complex containing the longest linker (*n* = 12) was comparable with that of cisplatin in both cell lines. This observation suggests that the mechanism underlying the biological action of the new dinuclear Pt^{II} complexes tested in the present work does not allow these complexes to overcome resistance mechanisms developed by cells against cisplatin. On the other hand, the experiments performed *in cellulo* strongly support the thesis that toxicity of long-chain dinuclear bifunctional *trans*-Pt^{II} complexes in tumor cells increases with the growing length of the aliphatic bridge linking Pt^{II} centers.

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Table 1 Summary of the effects of 1,1/t,t(dien)-*n* (*n* = 7,10,12) in comparison with cisplatin.

	$n = 7$	$n = 10$	$n = 12$	Cisplatin
DNA binding ($t_{50\%}$) (min)[a]	16 ± 1	15 ± 1	13 ± 1	120[d]
Preferential DNA binding sites[b]	G, A	G, A	G, A	GG, AG
% Interstrand cross-links/adduct[a]	56 ± 2	72 ± 3	91 ± 3	6[e]
Reduction of EtBr fluorescence	strong	strong	strong	medium
Circular dichroism at ~280 nm	increase at low r_b, decrease at high r_b	increase at low r_b, decrease at high r_b	increase at low r_b, decrease at high r_b	increase at low r_b, decrease at high r_b[f]
Toxicity[c] (IC_{50}, μM) after 72 h treatment of				
A2780 cells	6.0 ± 1.2	3.4 ± 0.2	2.3 ± 0.4	1.7 ± 0.4
A2780cisR cells	56.8 ± 5.1	40.0 ± 3.6	30.5 ± 4.2	23.9 ± 2.8

[a]See the text for details.
[b]Determined by transcription mapping.
[c][IC_{50} mean values (μM)], the results are expressed as mean ± SD of three independent experiments.
[d]Ref. [27].
[d]Ref. [20].
[e]Ref. [31].

DNA binding in cell-free media

One of the important early phases of the mechanism by which Pt compounds exert their anticancer activity is formation of adducts on nuclear DNA by these agents [24–26]. Hence, the ability of Pt complexes to coordinatively bind DNA and the rate of this binding are of great interest.

The first experiments were aimed at quantifying the binding of 1,1/t,t(dien)-*n* (*n* = 7,10,12) to mammalian DNA. Solutions of double-helical calf thymus (CT) DNA at a concentration of 32 μg mL^{-1} were incubated with the dinuclear Pt^{II} complexes at $r_i = 0.08$ in $NaClO_4$ (10 mM) at 37 °C. At various time intervals, aliquots of the reaction mixture were withdrawn and assayed for Pt complex bound to DNA. The amount of complexes bound to DNA increased with time, and all dinuclear Pt^{II} complexes were quantitatively bound after 6 h. The times at which the binding of 1,1/t,t(dien)-*n* complexes reached 50 % ($t_{50\%}$) were 16 ± 1, 15 ± 1, and 13 ± 1 min for *n* = 7,10,12, respectively. These results indicate that the rate of binding of the dinuclear Pt^{II} complexes tested in the present work to natural double-helical DNA is considerably higher than that of cisplatin ($t_{50\%}$ = ca. 120 min [27]). It implies that the somewhat reduced biological activity of 1,1/t,t(dien)-*n* (*n* = 7,10) in A2780 and A2780cisR cells is not due to an inherently slower rate of initial binding of these dinuclear Pt^{II} complexes to DNA.

One of the limiting factors that determine the cytotoxicity of Pt compounds is the nature of the conformational changes induced in DNA. Hence, data on DNA binding mode of Pt complexes are of great interest. In order to determine the character of DNA adducts of 1,1/t,t(dien)-*n* (*n* = 7,10,12) and structural characteristics of damaged DNA, several biochemical and biophysical methods have been applied. For these analyses, samples of DNA modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) at a preselected value of r_b (r_b is defined as the number of molecules of the Pt complex bound per nucleotide residue) prepared as described in the Experimental section were used. Thus, the analyses described below were performed in the absence of unbound (free) Pt complex.

In vitro RNA synthesis by T7 RNA polymerase on DNA templates [pSP73KB plasmid (2455 bp) linearized by NdeI restriction endonuclease] containing adducts of 1,1/t,t(dien)-*n* (*n* = 7,10,12) revealed (Fig. 2) that the major stop sites produced by these complexes were mainly at guanine residues. This result also suggests that the adducts formed by the dinuclear Pt^{II} complexes tested in the present work are able to inhibit RNA polymerase. Importantly, the sequence dependence of the inhibition of RNA synthesis by the adducts of 1,1/t,t(dien)-*n* was identical for all three dinuclear Pt^{II} complexes and was

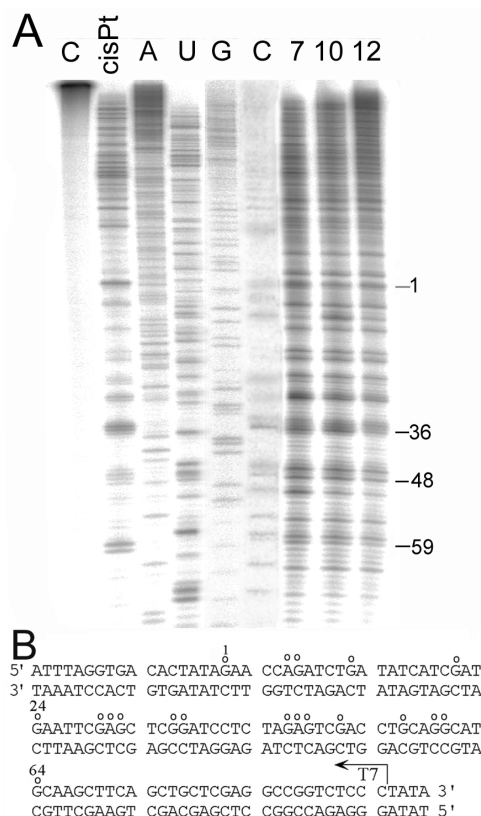

Fig. 2 Inhibition of RNA synthesis by T7 RNA polymerase on the fragment of pSP73KB plasmid linearized by NdeI modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) and cisplatin. A. Autoradiogram of 6 % polyacrylamide/8 M urea sequencing gel. Lanes: C - control, non-modified template; *cis*-Pt, 7, 10, 12 - the template modified by cisplatin, 1,1/t,t(dien)-*n*, *n* = 7,10,12 at r_b = 0.008, respectively; A, U, G, C - chain-terminated marker RNAs; B. Schematic diagram showing the portion of the nucleotide sequence of the template fragment used to monitor inhibition of RNA synthesis by the Pt^{II} complexes. The arrow indicates the start of the T7 RNA polymerase. (o), major stop signals (from Fig. 2A, lane 10). The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

considerably less regular than that by the adducts of cisplatin. The latter results indicate that the dinuclear Pt^{II} compounds tested in the present work form a greater variety of adducts with DNA and less regularly than cisplatin.

Bifunctional Pt compounds that covalently bind to DNA form various types of interstrand and intrastrand CLs. Considerable evidence suggests that the antitumor efficacy of bifunctional Pt compounds is the result of the formation of these lesions, but their relative efficacy remains unknown. We quantified the interstrand cross-linking efficiency of 1,1/t,t(dien)-*n* (*n* = 7,10,12) in pSP73KB plasmid linearized by EcoRI by gel electrophoresis under denaturing conditions [11] (Fig. 3). The interstrand cross-linking efficiencies (defined as the number of interstrand CLs per one adduct of the Pt complex [11] of 1,1/t,t(dien)-*n* complexes reached) were 56 ± 2, 72 ± 3, and 91 ± 3 % for *n* = 7,10,12, respectively. These results indicate that DNA interstrand cross-linking efficiency of 1,1/t,t(dien)-*n* (*n* = 7,10,12) increased with growing length of the linker. In addition, these results demonstrate that the most prevalent adducts formed in double-helical DNA by these dinuclear Pt^{II} complexes are interstrand CLs, which is in contrast to mononuclear cisplatin, which forms only 6 % interstrand CLs [20], and its major adducts are intrastrand CLs between neighboring base residues [21].

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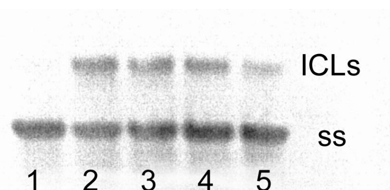

Fig. 3 The formation of interstrand cross-links (ICLs) by 1,1/t,t(dien)-*n* (*n* = 7,10,12) and cisplatin in pSP73KB DNA. Autoradiograms of the denaturing 1 % agarose gels of linearized DNA which was 3'-end-labeled; the interstrand cross-linked DNA appears as the top bands (ICLs) migrating on the gels more slowly than the single-stranded (ss) DNA (contained in the bottom bands). Plasmid linearized by EcoRI was nonmodified (lane 1), modified by 1,1/t,t(dien)-*n*, *n* = 7,10,12 at r_b values of 1×10^{-4} (lanes 4,3,2, respectively), or by cisplatin at $r_b = 0.001$ (lane 5).

Binding of ethidium bromide (EtBr) to DNA by intercalation is blocked in a stoichiometric manner by formation of the bifunctional adducts of a series of Pt complexes including cisplatin, which results in a loss of fluorescence intensity. Modification of DNA by 1,1/t,t(dien)-*n* (*n* = 7,10,12) or cisplatin resulted in a decrease of EtBr fluorescence (Fig. 4). The decrease caused by the adducts of the dinuclear Pt^{II} complexes tested in the present work was markedly more pronounced than that induced by the DNA adducts of cisplatin at equivalent r_b values. In addition, this effect on EtBr fluorescence increased with the growing length of the aliphatic bridge linking Pt^{II} centers. The results of these experiments (Fig. 4) suggest that the conformational distortion induced in DNA by the adducts of 1,1/t,t(dien)-*n* (*n* = 7,10,12) extends over a markedly higher number of base pairs around the platination sites as in the case of the adducts of cisplatin. A plausible explanation of the distinct behavior of DNA adducts of the dinuclear Pt^{II} complexes tested in the present work (Fig. 4) is that these complexes preferentially form long-range CLs similarly as other di/trinuclear Pt^{II} complexes do [9,18,19,28–30].

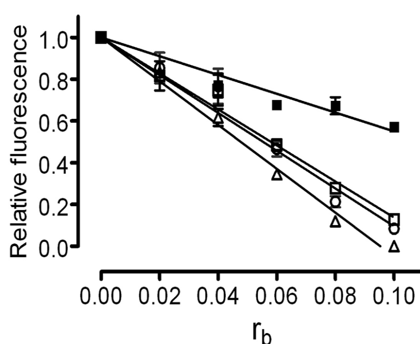

Fig. 4 Dependencies of EtBr fluorescence on r_b for double-helical CT DNA modified by Pt complexes. Data points were measured in triplicate and varied on average ±3 % from their mean. Fluorescence of untreated DNA was arbitrarily set at unity. Symbols: (□), 1,1/t,t(dien)-7; (○), 1,1/t,t(dien)-10; (△), 1,1/t,t(dien)-12; (■), cisplatin. Values shown in the graph are the means (±SEM) of three separate experiments.

Circular dichroism (CD) spectral characteristics for CT DNA modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) to values of r_b in the range 0.025–0.3 (Fig. 5) were determined. Upon binding of these compounds to CT DNA, the conservative CD spectra normally found for DNA in the canonical B-conformation considerably transform at wavelengths below 300 nm. There was a slight increase in the intensity of the positive band around 280 nm if DNA was modified at relatively lower values of r_b (Fig. 5). At higher levels of the modification (r_b > ~0.6), the intensity of this CD band began to decrease, this

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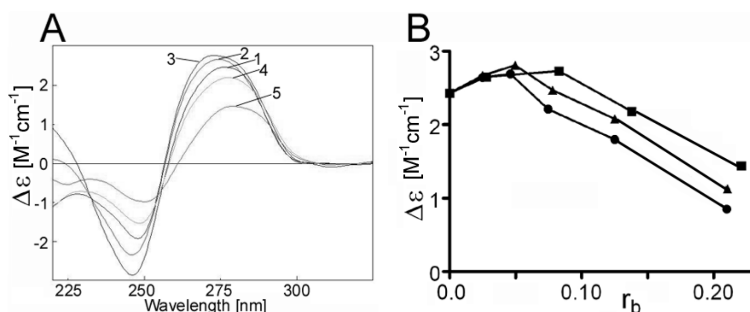

Fig. 5 A. CD spectra of double-helical CT DNA modified by 1,1/t,t(dien)-7. CD spectra were recorded for DNA at the concentration of 32 μg mL^{-1} in 10 mM $NaClO_4$ with 10 mM Tris-HCl, pH 7.0. Curves: 1, r_b = 0, control (unplatinated) DNA; 2, r_b = 0.03; 3, r_b = 0.08; 4, r_b = 0.14; 5, r_b = 0.22. B. Changes in the CD spectra of DNA at λ_{max} around 280 nm (at the wavelength at which the maximum of the positive CD band around 280 nm occurred) induced by the binding of 1,1/t,t(dien)-*n*, *n* = 7 (■), 10 (▲), and 12 (●).

effect increased with the growing length of the aliphatic bridge linking Pt^{II} centers. Thus, the dinuclear Pt^{II} complexes tested in the present work affected the CD spectra of CT DNA in a way similar to cisplatin under identical conditions [31]. We interpreted CD spectra of DNA modified by the dinuclear Pt^{II} complexes on the basis of the analogy with the changes in the CD spectra of DNA modified by cisplatin and clinically ineffective transplatin [31]. It might be suggested that the binding of 1,1/t,t(dien)-*n* (*n* = 7,10,12) affects the DNA conformation such that the character of the conformational changes induced in DNA corresponds at lower levels of DNA platination to nondenaturational conformational alterations. At higher levels of DNA platination, the character of the conformational changes induced in DNA corresponds rather to denaturational alterations [31]. Analysis of DNA modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) also revealed that efficiency of these dinuclear Pt^{II} complexes to affect DNA conformation increased with the growing length of the aliphatic bridge linking Pt^{II} centers.

Mammalian CT DNA was modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) to various r_b values and melting curves were recorded in the medium of a relatively high ionic strength containing 0.1 M Na^+ (Fig. 6). The effect on melting temperature (t_m) was dependent on the amount of Pt complex bound (Fig. 6). The modification of DNA by the dinuclear Pt^{II} complexes at lower r_b values (up to r_b ~0.08) resulted in a slight increase of t_m. In contrast, at higher levels of the modification, t_m began to decrease. This decrease of t_m was most pronounced for the dinuclear Pt^{II} complex containing the longest aliphatic bridge linking Pt^{II} centers (*n* = 12).

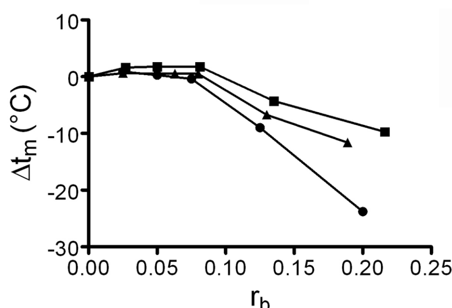

Fig. 6 Plots of Δt_m values of CT DNA modified by 1,1/t,t(dien)-*n* on r_b. Symbols: (■), *n* = 7; (▲), *n* = 10; (●), *n* = 12. The t_m values were measured in a medium containing 0.1 M $NaClO_4$ plus Tris-HCl (1 mM, pH 7.4) and EDTA (0.1 mM) (Δt_m is defined as the difference between the t_m values of platinated and unmodified DNAs).

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Previously, three major factors have been invoked to account for the thermal stability of DNA modified by Pt^{II} complexes capable of DNA cross-linking and the observed change in t_m of DNA as a consequence of its platination reflects the relative proportion and contribution of these three factors [32]. These major factors are (i) a destabilizing effect of conformational distortions due to the formation of CLs induced in DNA by Pt coordination; (ii) stabilizing effects of DNA interstrand CLs, which prevent dissociation of DNA strands; (iii) the positive charge on the Pt^{II} centers (introduction of a positive charge into the DNA molecule, e.g., by binding of positively charged ligands such as Pt^{II} moieties of Pt antitumor compounds, results in a stabilization of the DNA duplex by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands). However, it has been shown [33] that cations present in the medium also stabilize DNA against thermal denaturation by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands. Hence, the stabilization effect of the positive charge on the Pt^{II} centers practically disappears at sufficiently high concentrations of the cations. Thus, at high ionic strength (0.1 M Na^+) when the electrostatic stabilizing effects of the dinuclear Pt^{II} complexes tested in the present work are markedly weakened, the value of t_m is affected by destabilizing effects of conformational distortions induced by lesions of the Pt complexes and the stabilizing effects of interstrand CLs.

Therefore, the observation that the t_m values of DNA modified by 1,1/t,t(dien)-*n* (*n* = 7,10,12) considerably decreased at higher r_b values is consistent with the occurrence of conformational alterations induced in DNA by these complexes, which destabilize the duplex. In other words, at higher Na^+ concentrations, destabilization of DNA resulting from conformational distortions induced by 1,1/t,t(dien)-*n* (*n* = 7,10,12) overcompensates the stabilization effects of DNA interstrand CLs. Interestingly, the decrease of t_m values observed at higher r_b values increased with the growing length of the aliphatic bridge linking Pt^{II} centers (Fig. 6) with the concomitant increase of the interstrand cross-linking efficiency (vide supra). Hence, the results of DNA melting experiments are consistent with the thesis that the growing length of the aliphatic bridge linking Pt^{II} centers promotes more distorting conformational alterations induced in DNA by the dinuclear Pt^{II} complexes tested in the present work.

Reactions with reduced glutathione

Pt^{II} compounds have a strong thermodynamic preference for binding to S-donor ligands, such as thiolates [34,35]; hence, before Pt^{II} drugs reach the DNA in the nucleus of tumor cells, or even after they bind to DNA, they may still react with various compounds including S-containing molecules [36,37]. These reactions are generally believed to play a role in mechanisms underlying tumor resistance to Pt compounds, their inactivation, and side effects. Therefore, interest in the interactions of Pt antitumor drugs with S-containing molecules of biological significance has recently markedly increased [37].

In the present work, we investigated, using UV absorption spectrophotometry, irreversible binding of GSH with 1,1/t,t(dien)-7 in comparison with 1,1/t,t-6 following exactly the procedure outlined by Dabrowiak et al. [38]. The dinuclear Pt^{II} complexes at a concentration of 32 μM were incubated with 16 mM GSH (these concentrations of Pt complex and GSH and the ratio of thiol to drug (500:1) represent the physiologically relevant values [38]) at 37 °C in 4.6 mM NaCl plus 100 mM $NaClO_4$ pH 6.0 as described in the Supplementary Information. Figure 7 shows UV absorption (at 260 nm) of the Pt complexes and GSH as a function of time, with the absorptions of GSH and Pt complex alone subtracted. The $t_{1/2}$ (half-times of the reactions, which mainly result in the formation of Pt–S bonds) were 81.8 ± 1.2 for 1,1/t,t(dien)-7 (one-phase exponential association) and 64.4 ± 1.5 and 6.4 ± 0.6 min for reaction 1,1/t,t-6 with GSH (two-phase exponential association). Thus, 1,1/t,t(dien)-7 reacted with GSH more slowly than 1,1/t,t-6. 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 [24,39–41]. Further studies are warranted to dissect what part of the cytotoxicity of 1,1/t,t(dien)-7 is unfavorably affected by its reaction with GSH.

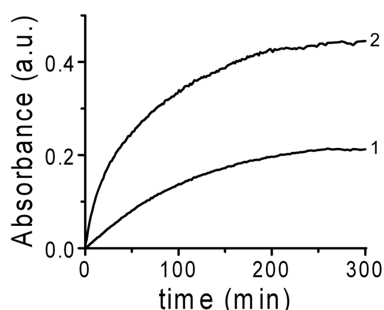

Fig. 7 UV absorbance associated with the reaction of 1,1/t,t(dien)-7 and 1,1/t,t-6, with GSH. Absorbance at 260 nm is shown as a function of time for a 300 min incubation at 37 °C of the Pt complexes at the concentration of 32 μM with 16 mM GSH in the medium of 4.6 mM NaCl plus 0.1 mM Tris-HCl buffer, pH 6.8, in the dark. The curves represent absorbances (260 mm) of a solution containing Pt complex plus GSH from which absorbances yielded by GSH and Pt complex alone were subtracted. Curves: 1, 1,1/t,t(dien)-7; 2, 1,1/t,t-6.

Results describing irreversible binding of GSH to 1,1/t,t-6 and 1,1/t,t(dien)-*n*, *n* = 7,10,12 showed that the absorbance at 260 nm determined for the reaction of GSH with 1,1/t,t(dien)-7 was approximately 2-fold lower than that determined for the reaction of GSH with 1,1/t,t-6 (e.g., absorbances at 260 nm after 300 min reaction with GSH of 1,1/t,t(dien)-7 and 1,1/t,t-6 were 0.213 and 0.445, respectively, Fig. 7). The absorbance at 260 nm reflects the presence of Pt–S and disulfide bonds [38,42]. Hence, our results (Fig. 7) can be interpreted to mean that, under conditions of our experiments, the major products of the reaction of this tripeptide with 1,1/t,t-6 contained a 2-fold higher amount of GSH per Pt atom than those afforded by the reaction of GSH with 1,1/t,t(dien)-7. *Trans*-oriented dinuclear bifunctional Pt^{II} compounds containing NH_3 non-leaving groups (having the general structure [{*trans*-$PtCl(NH_3)_2\}_2$-μ-Y]$^{n+}$ where Y = linker, such as $NH_2(CH_2)_6NH_2$, spermidine, etc., are susceptible to decomposition [8,22,30]. This decomposition is a consequence of substitution of the Pt–Cl bond by a *trans*-labilizing S donor, which results in the loss of the linker [22]. Thus, the principal and initial products of the reaction of 1,1/t,t-6 with GSH are the disubstituted dinuclear complexes [{*trans*-$Pt(SG)(NH_3)_2\}_2$-μ-$NH_2(CH_2)_6NH_2]^{2+}$ [22,43,44]. The subsequent liberation of the alkanediamine linker due to its *trans*-labilization from the dinuclear Pt^{II} moiety results in mononuclear species with a 1:2 Pt:GSH stoichiometry or S-bridged Pt species. The 2-fold lower absorption at 260 nm observed for the reaction of GSH with 1,1/t,t(dien)-7 (Fig. 7) compared with the reaction of GSH with 1,1/t,t-6 may imply that the amount of products afforded by the reaction of GSH with 1,1/t,t(dien)-7 contained only the disubstituted dinuclear complexes [{*trans*-$Pt(SG)(dien)_2\}_2$-μ-$(CH_2)_7]^{2+}$ which were no longer transformed to mononuclear complexes with two *trans*-GSH ligands or to bridged Pt species (with the 1:2 Pt:GSH stoichiometry). The dien ligand cannot be readily displaced from the Pt by S-containing ligands because it is stabilized by a strong chelate effect as evidenced by the finding that the major product of the reaction of mononuclear $[PtCl(dien)]^+$ with excess GSH at pH < 7 is $[Pt(dien)GS]^+$ [34]. Thus, at most we expect only one GSH to be bound to each Pt atom in the dinuclear [{*trans*-$Pt(SG)(dien)\}_2$-μ-$(CH_2)_7]^{2+}$ (in contrast with [{*trans*-$Pt(SG)(NH_3)_2\}_2$-μ-$NH_2(CH_2)_6NH_2]^{2+}$) where the linker can be labilized. The chelating and bulkier ligands in 1,1/t,t(dien)-*n* could reduce the deactivation of Pt compounds caused by thiol-containing biomolecules such as reduced GSH as already demonstrated for other dinuclear and bifunctional antitumor Pt^{II} complexes [45,46]. In aggregate, the steric properties of the amine ligands appear to be the dominating factor in determining the substitution kinetics of 1,1/t,t-6 and 1,1/t,t(dien)-7 when species with the 1:2 Pt:GSH stoichiometry are formed. Thus, our results support the thesis that the dinuclear structure of 1,1/t,t(dien)-7 remains stable in the presence of S-containing compounds without undergoing chemical degradation as previously observed for some di/trinuclear bifunctional Pt^{II} complexes [8,22,30]. Such a favorable property may contribute

to reduce side effects and increase therapeutic efficacy of the di/trinuclear bifunctional Pt^{II} agents [8,47]. This thesis is consistent with the findings demonstrating that, for instance, 1,1/t,t-4 exhibited a considerably worse toxicity in ovarian tumor cell lines sensitive to cisplatin (IC_{50}) than analogous {$[Pt(dien)Cl]_2$-alkane} complexes. More specifically, the cytotoxicity of 1,1/t,t-4 was more than one order of magnitude worse than that exhibited by cisplatin [11] whereas {$[Pt(dien)Cl]_2$-alkane} complexes tested in the present work exhibited cytoxicity in ovarian tumor cell lines sensitive to cisplatin which was comparable to cisplatin (Table 1).

CONCLUSIONS

Trans-oriented dinuclear bifunctional Pt^{II} complexes containing aliphatic linker chains represent prototypical compounds of a class of polynuclear Pt am(m)ine complexes that exhibit antitumor and DNA-binding properties that are significantly different from the mononuclear anticancer Pt complexes based on cisplatin. The chemistry and biological activity of this class of Pt complexes may be modulated by the geometry and number of leaving groups in the coordination sphere as well as the nature of the chain linking the Pt centers. Varying the length of the linker may affect the distance and spatial configuration of the reactive groups in these dinuclear Pt^{II} compounds, hence also their DNA binding mode and consequently biological effect.

Systematic studies described in this report of the effect of the length of the linker in particular for the *trans*-oriented dinuclear bifunctional Pt^{II} complexes containing longer aliphatic linker chains (heptane and longer alkanes) have revealed that the growing length of the aliphatic bridge linking Pt^{II} centers promotes more distorting conformational alterations induced in DNA by the dinuclear Pt^{II} complexes. This result correlates with the increase of toxicity of these long-chain dinuclear bifunctional *trans*-Pt^{II} complexes in tumor cells with the growing length of the aliphatic bridge linking Pt^{II} centers.

In general, *trans*-oriented dinuclear bifunctional Pt^{II} complexes containing aliphatic linker chains are susceptible to decomposition by S-containing compounds. This decomposition is responsible for tumor resistance to Pt compounds, inactivation of these metallodrugs, and side effects. The results of our studies of the effects of {$[Pt(dien)Cl]_2$-alkane} described in the present report support the thesis that the steric properties and chelate effects of the amine ligands in this class of Pt^{II} complexes appear to be the dominating factors in determining whether these antitumor metallodrugs remain stable in the presence of S-containing compounds without undergoing chemical degradation. This enhanced stability represents a favorable property which undoubtedly contributes to reduce side effects and increase therapeutic efficacy of the dinuclear bifunctional Pt^{II} agents.

SUPPLEMENTARY INFORMATION

Experimental details are available online (http://dx.doi.org/10.1351/PAC-CON-12-02-08).

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