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Highly Efficient DNA Compaction Mediated by an In Vivo Antitumor-Active Tetrazolato-Bridged Dinuclear Platinum(II) Complex

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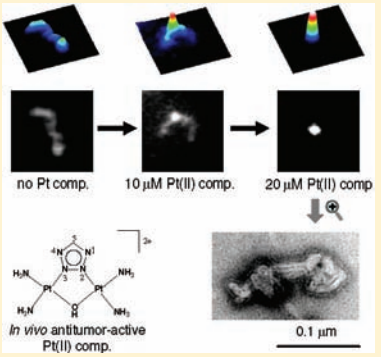
ABSTRACT: We investigated the effects of antitumor-active tetrazolato-bridged dinuclear platinum(II) complexes $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-tetrazolato-}N^1,N^2)]^{2+}$ (**1**) and $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-tetrazolato-}N^2,N^3)]^{2+}$ (**2**) on the higher-order structure of a large DNA molecule (T4 phage DNA, 166 kbp) in aqueous solution through single-molecule observation by fluorescence microscopy. Complexes **1** and **2** cause irreversible compaction of DNA through an intermediate state in which coil and compact parts coexist in a single DNA molecule. The potency of compaction is in the order **2** > **1** ≫ cisplatin. Transmission electron microscopic observation showed that both complexes collapsed DNA into an irregularly packed structure. Circular dichroism measurements revealed that the dinuclear platinum(II) complexes change the secondary structure of DNA from the B to C form. These characteristics of platinum(II) complexes are markedly different from those of the usual condensing agents such as spermidine(3+) and $[Co^{III}(NH_3)_6]^{3+}$. The ability to cause DNA compaction by the platinum(II) complexes is discussed in relation to their potent antitumor activity.



INTRODUCTION

The approval of *cis*-diamminedichloridoplatinum(II) (cisplatin) for clinical use in the 1970s prompted a search for new platinum coordination compounds with even greater efficacy.[1−5] Platinum-based drugs, including cisplatin and a few derivatives, are now widely used and established chemotherapeutic agents. Although they have some clinical disadvantages, such as serious side effects and acquired resistance, the high therapeutic efficacies of platinum-based drugs have inspired the development of next-generation agents that are effective against chemotherapy-resistant cancers with fewer side effects. Azolato-bridged dinuclear platinum(II) complex cations such as $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-pyrazolato})]^{2+}$ and $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-1,2,3-triazolato-}N^1,N^2)]^{2+}$ are known to be highly cytotoxic toward a cisplatin-resistant cell line, as well as several human tumor cell lines, probably because their biological effects are different from those of cisplatin.[6−8] Very recently, Komeda et al. reported that two linkage isomers of tetrazolato-bridged dinuclear platinum(II) complexes, $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-tetrazolato-}N^1,N^2)]^{2+}$ (**1**) and $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-tetrazolato-}N^2,N^3)]^{2+}$ (**2**), show high in vitro cytotoxicity in a human tumor cell line, and **2** exhibits markedly high in vivo antitumor efficacy toward xenografted tumors of PANC-1 pancreatic cancer.[9] The chemical structures of **1** and **2** are shown in Figure 1.

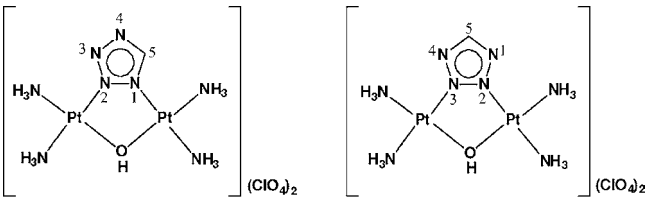


Figure 1. Chemical structures of complexes **1** and **2**.

It is generally accepted that platinum-based clinical anticancer drugs form coordinative adducts on genomic DNA, such as 1,2-intrastrand cross-links, to interfere with transcription and/or DNA replication, which eventually leads to apoptotic cell death.[3,10] Accordingly, the therapeutic effects of platinum-based drugs are thought to be triggered by this cross-linking. X-ray crystallography[11,12] and 2D NMR[13] techniques have revealed the local configurations of cisplatin cross-linking sites on DNA that are severely distorted relative to normal B-form DNA. Thus, the outcomes of drug−DNA

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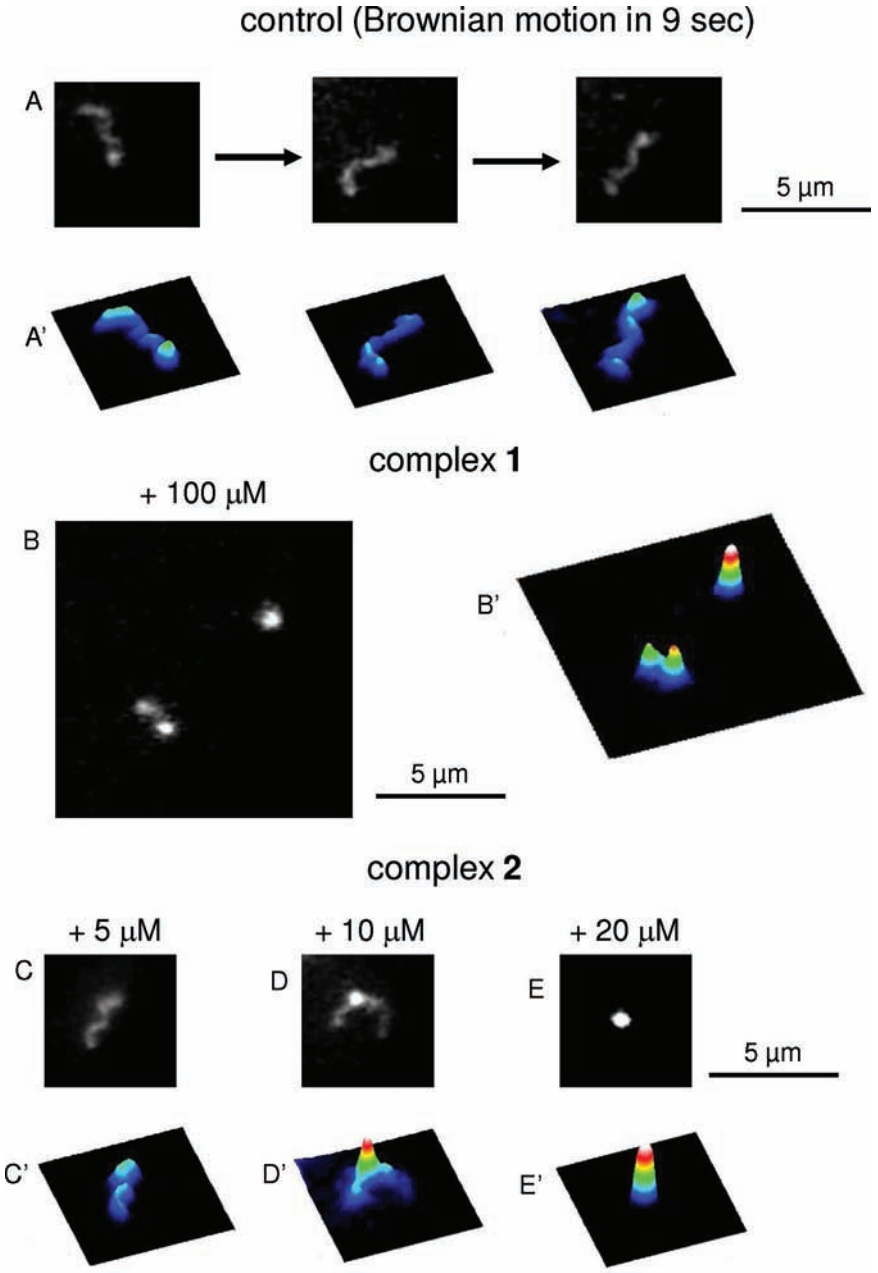

Figure 2. Fluorescence microscopy observation of individual T4 DNA molecules moving freely in solution (A–E) and corresponding quasi-3D profiles of the fluorescence intensity (A′–E′). (A) Time-successive fluorescence image of a single DNA molecule exhibiting Brownian motion in a buffer solution. The time interval between the first and last flames is 9 s. (B) In the presence of 100 μM **1**, the partial-globule and globule states coexist. In the presence of (C) 5, (D) 10, and (E) 20 μM **2**. These images were obtained within 30 min after the addition of the complexes.

interaction can be used to elucidate essential aspects of the mechanism of action and provide clues to the discovery of more efficient DNA-targeted anticancer drugs. So far, X-ray crystallography and 2D NMR techniques have been shown to be very useful for determining local configurations and binding modes with short DNA fragments such as oligonucleotide. It is well-known that such short DNA behaves as a rigid rod and cannot bend easily, while genomic DNA is a very long polymer (up to several centimeters in length) and thus behaves as a flexible chain. We have found that a long duplex DNA molecule larger than 100 kbp exhibits an on/off change in conformation from a coil state to a folded compact state upon the addition of various condensing agents based on the results of single-molecule observation in solution using fluorescence microscopy.[14] In a related study, Liu et al. reported that the DNA length is an important factor that governs drug–DNA interaction based on the results of atomic force microscopy observations of cisplatin–DNA binding.[15] Therefore, DNA larger than 100 kbp may be a more realistic model for platinated DNA in living cells.

Complexes **1** and **2** are promising drug candidates, but their molecular mechanisms are not yet known. Genomic DNA is a likely target because azolato-bridged dinuclear platinum(II) complexes cross-link two adjacent nucleobases with minimal kinking of the DNA double helix; the hydroxo anion acts as a leaving group, which enables bifunctional coordinative binding.[6,16] In the present study, we investigated the effects of **1** and **2** on the higher-order structure of large DNA through single-

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molecule observation to gain insight into the mechanism of their anticancer effects. For this purpose, we chose T4 phage DNA (166 kbp), the size of which is close to that of the chromatin loop domain (100−200 kbp).[17]

EXPERIMENTAL SECTION

Materials. T4 phage DNA (166 kbp, contour length 57 μm) was purchased from Nippon Gene (Toyama, Japan). The fluorescent cyanine dye YOYO-1 (1,1′-[1,3-propanediylbis[(dimethylimino)-3,1-propanediyl]]bis[4-[(3-methyl-2(3*H*)-benzoxazolylidene)methyl]]-tetraiodide quinolinium) was purchased from Molecular Probes Inc. (Eugene, OR). The antioxidant 2-mercaptoethanol (2-ME) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Tetrazolato-bridged complexes **1** and **2** were synthesized as described in the literature.[9] Other chemicals were of analytical grade and were obtained from various commercial sources.

Direct Observation of the Change in the Conformation of DNA in Aqueous Solution by Fluorescence Microscopy. T4 phage DNA was dissolved in a 10 mM Tris−HCl buffer and 4% (v/v) 2-ME at pH 7.6 in the presence of various concentrations (5−100 μM) of **1** or **2**. Measurements were conducted at a low DNA concentration (0.1 μM in nucleotide units). To visualize individual DNA molecules by fluorescence microscopy, the cyanine dye YOYO-1 was added to the DNA solution after incubation with these complexes. YOYO-1 is known to bind to double-stranded DNA through base-pair intercalation and has been reported to show a strong increase in fluorescence upon binding to DNA.[18] To minimize the effect of YOYO-1 on DNA−drug interaction, a low concentration of YOYO-1 (0.05 μM) was added to the samples just before the single-DNA observation. Fluorescent DNA images were obtained using a microscope (Axiovert 135 TV; Carl Zeiss, Oberkochen, Germany) equipped with a 100× oil-immersion objective lens and a highly sensitive EBCCD camera (Hamamatsu Photonics, Shizuoka, Japan), which made it possible to record images on DVD. The video images were analyzed with Cosmos image-analysis software (Library, Tokyo, Japan).

Electron Microscopic Observation. Samples used for electron microscopy were mounted on carbon-coated copper grids (no. 200), negative-stained with 2% ammonium molybdate, and observed with a transmission electron microscope (model JEM-1400EX; JEOL, Tokyo, Japan) at 100 kV. The DNA concentration was 0.1 μM.

Circular Dichroism (CD) Spectroscopic Measurements. CD spectra of T4 DNA modified by **1**, **2**, and cisplatin were measured at 25 °C in 10 mM Tris−HCl buffer (pH 7.6) on a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan). The DNA concentration was 30 μM. The concentrations of the platinum(II) complexes were 0, 5, and 10 μM. The cell path length was 1 cm. Spectra were recorded in the range of 220−340 nm at a scan rate of 50 nm/min.

RESULTS

To investigate the effects of cationic platinum(II) complexes **1** and **2** on the higher-order structure of DNA, we performed fluorescence microscopy observations of DNA in the presence and absence of the platinum(II) complexes. Figure 2 shows representative fluorescence images of individual DNA molecules in aqueous solution together with the corresponding quasi-3D profiles of the fluorescence intensity. In order to avoid multimolecular aggregation, observations were performed at a low DNA concentration (0.1 μM). In the absence of both complexes, DNA molecules exist in an elongated coil conformation (Figure 2A) and exhibit translational and intramolecular Brownian motion in aqueous solution. Upon the addition of **1** or **2** to the DNA solution, individual DNA molecules undergo a structural transition from a coil state (Figure 2A,C) to a compact-globule state (Figure 2B,E) via a partial globule state (Figure 2B,D) in which elongated and shrunken parts coexist in a single DNA molecule. These observations clearly indicate that both **1** and **2** have a significant effect on the higher-order structure of DNA. It is noted that the partial-globule and globule structures are stable even at high salt concentration; these structural changes were irreversible, as revealed by conformations of platinated DNA before and after the addition of salt. As shown in Figure 3, there was no marked

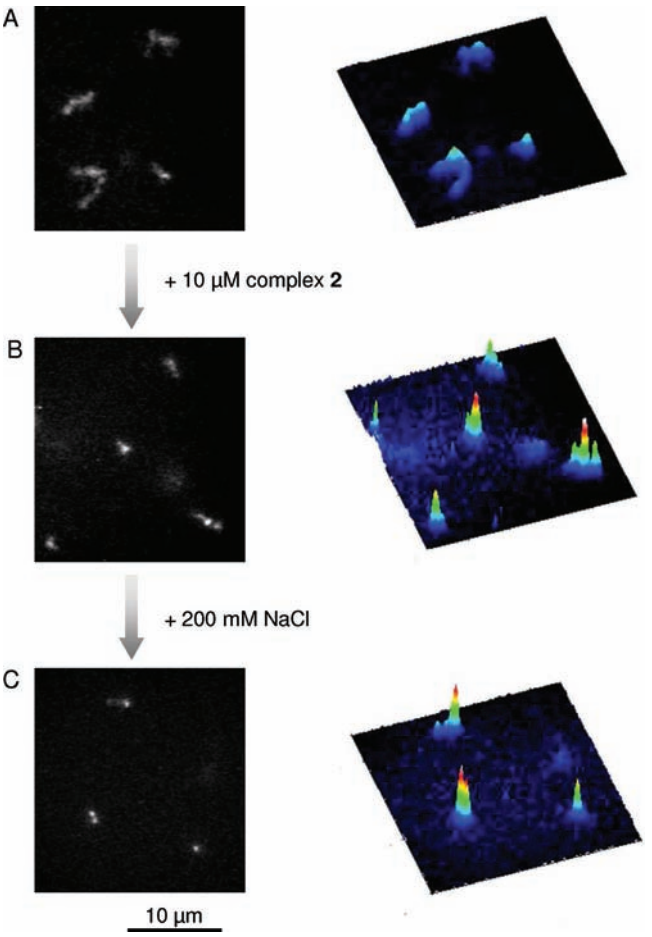

Figure 3. Fluorescence microscopy images of T4 DNA observed in (A) a buffer solution and (B) 10 μM **2**. The image was obtained within 30 min after the addition of **2**. (C) 1 h after the addition of 200 mM NaCl to part B.

change in their conformations even 1 h after the addition of 200 mM NaCl. This may be due to the direct irreversible binding of these complexes to DNA.

Figure 4 shows the distribution of the long-axis length of DNA, where the conformation is classified as a coil, partial globule, or globule based on the time-successive fluorescence microscopy observations (Figure 2). In the presence of 10 μM **2**, most of the DNA molecules exist in a partial-globule state (Figure 4D), while DNA molecules were in an elongated coil state under the addition of the same amount of **1** (Figure 4C). By the addition of 20 μM **2**, 94% of the DNA molecules turned into a compact-globule conformation, while even in the presence of 50 μM **1**, only 8% of the DNA molecules were in the globule state. For comparison, Figure 4B shows the size distribution with 100 μM cisplatin. It is noted that no compacted DNA was observed even in the presence of 100 μM cisplatin (Figure 4B). These results clearly demonstrate

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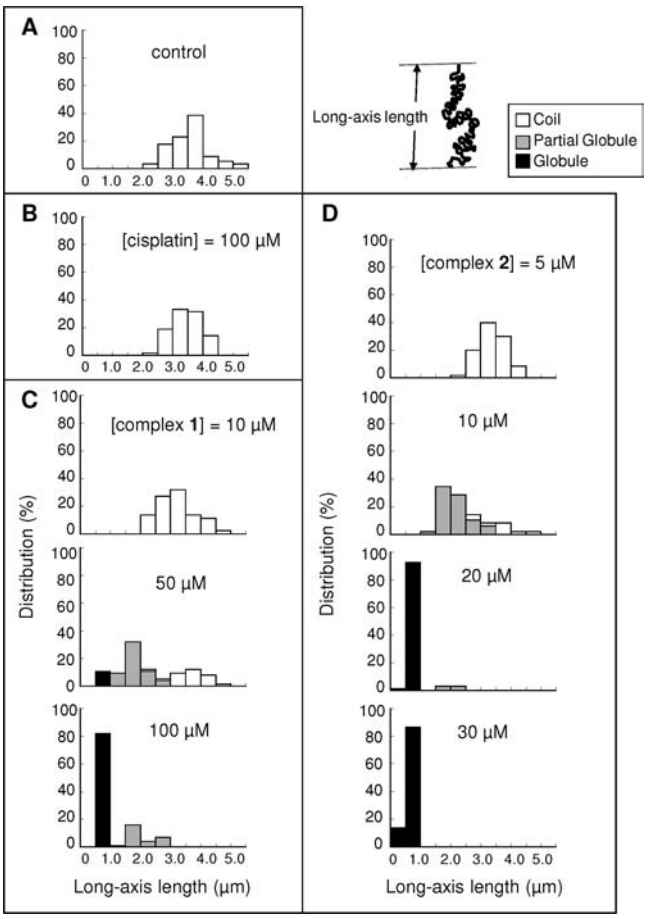

Figure 4. Comparison of the distribution of the long-axis length of DNA in solution together with assignment of the conformational characteristics in DNA images: (A) in a buffer solution; (B) cisplatin; (C) **1**; (D) **2**. (Top right) Illustration showing a schematic representation of a fluorescent image of DNA.

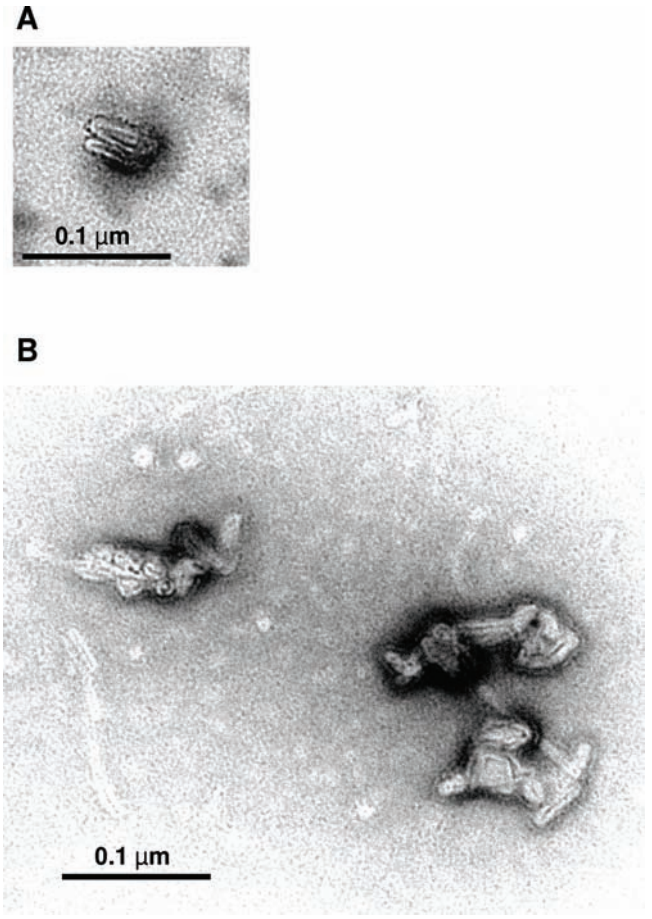

Figure 5. TEM images of compacted DNA particles in the presence of (A) 100 μM **1** and (B) 20 μM **2**. These images were obtained within 1 h after the addition of the complexes.

that **2** has a much more potent effect on the higher-order structure of DNA than **1** and cisplatin.

The detailed morphology of compacted DNA was investigated using transmission electron microscopy (TEM). Irregularly packed DNA particles were captured in the presence of **1** (Figure 5A; 100 μM) or **2** (Figure 5B; 20 μM), where the size is on the order of 0.1 μm. While there were no specific differences in morphology between these compacted DNAs, these platinum(II) complexes had different potencies in the induction of DNA compaction.

To further interpret the changes in the conformation of DNA, we performed CD measurements of T4 DNA with platinum(II) complexes (Figure 6). Observations were performed immediately after the addition of complexes. In the presence of **1** or **2**, drastic changes in the overall CD spectrum of DNA were observed (Figure 6A,B). Figure 6C shows the CD spectra in the presence of cisplatin for comparison, indicating almost no change in the CD spectra. A previous study reported that cisplatin induced a B- to A-like conformational change and that the change became observable after several hours of incubation.[19] Thus, the effect of **1** and **2** on the secondary structure of DNA is more significant than that of cisplatin, at least for its kinetics.

The profile of CD spectra for DNA in the presence of **1** (Figure 6A) closely resembles that in the presence of **2** (Figure 6B). Simultaneous changes in both the positive and negative Cotton effects were noted: a significant decrease in the positive Cotton effect at around 280 nm and a small decrease in the negative Cotton effect at around 245 nm were observed in both complexes. These results show that DNA undergoes a transition in its secondary structure from the B to C form upon the addition of **1** or **2** and that **2** appears to be more effective at inducing this B-to-C transition. C-form DNA has been considered as a variant of the B form with a widened minor groove, which may involve the action of proteins on DNA including DNA–DNase 1 interaction.[20]

■ DISCUSSION

Over the past few decades, extensive studies on local drug–DNA binding and conformational changes have been performed with a relatively short DNA oligomer using X-ray crystallography and NMR to help clarify the mechanism of action, which could lead to the development of more efficient drugs. In contrast, little is known about the effects on the higher-order structure of DNA larger than 100 kbp. In the present study, T4 DNA (166 kbp) was chosen as a giant genomic DNA. As mentioned above, we demonstrated that two linkage isomers, **1** and **2**, cause compaction of DNA (Figure 2) accompanied by changes in the secondary structure of DNA from the B to C form (Figure 6), using the same large DNA. The potency of compaction is in the order **2** > **1** ≫ cisplatin (Figure 4).

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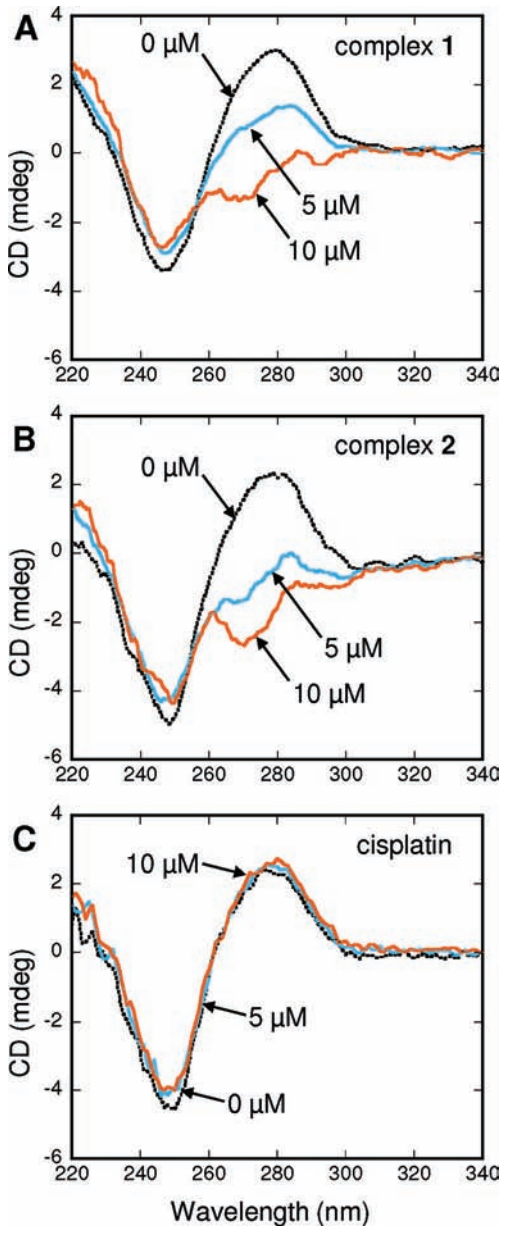

Figure 6. CD spectra of T4 DNA in the presence of (A) **1**, (B) **2**, and (C) cisplatin. The DNA concentration was 30 μM. The drug/nucleotide molar ratios were 0, 0.17, and 0.33 for all drugs. Spectra were obtained immediately after the addition of **1**, **2**, or cisplatin.

Interestingly, it has been reported that both **1** and **2** exhibit high cytotoxicity toward the human NSCLC cell line H460; the IC_{50} values for **1**, **2**, and cisplatin are 23.5 ± 1.6, 9.2 ± 0.6, and 23.1 ± 1.8 μM, respectively, indicating that **2** is indeed twice as cytotoxic as cisplatin and **1**.[9] Moreover, **2** exhibits high in vivo antitumor efficacy against nonsmall-cell lung cancer and markedly high efficacy against pancreatic cancer.[9,21] These facts suggest that the ability to change the higher-order and secondary structures of DNA may be related to the antitumor efficacy.

Irreversible DNA compaction induced by **2** having two positive charges is noted (Figure 3). Generally, the binding of charged ligands to DNA is sensitive to the salt concentration. A previous study showed that the nonantitumor divalent cation tetraammineplatinum(II), for which coordination binding cannot be anticipated because the platinum(II) coordination sphere is filled with inert nitrogen-donor ligands, induces DNA compaction, but the change in the higher-order structure is reversible upon the addition of salt.[22] Therefore, irreversible DNA compaction may be mediated through coordinative and relatively strong noncoordinative interactions, both of which may contribute to its potent antitumor activity. With regard to the route toward DNA compaction, the change in the higher-order structure of a long DNA molecule induced by platinum(II) complexes is characterized as a gradual shrinking; the long-axis length of DNA gradually decreases while maintaining a distribution profile with a *single peak* (Figure 4). Furthermore, platinum(II)-complex-induced DNA compaction was accompanied by significant CD changes (Figure 6), and irregularly packed DNA particles were observed in TEM observations (Figure 5). These behaviors of DNA compaction are markedly different from those induced by effective trivalent condensing agents, such as spermidine and $[Co^{III}(NH_3)_6]^{3+}$. It has been reported that spermidine and $[Co^{III}(NH_3)_6]^{3+}$ induce DNA condensation without any changes in the CD spectrum using a rather large DNA, T7 DNA (40 kbp)[23] or λ DNA (48 kbp).[24] TEM observations showed that these trivalent cationic compounds condense DNA into a well-ordered toroidal structure.[23−29] These findings demonstrate that platinum(II) complexes induce DNA compaction in a manner different from that of typical condensing agents.

Figure 7 schematically illustrates the difference in the route toward DNA compaction between **2** and spermidine. Spermidine induces DNA compaction in an all-or-none manner (Figures 7F,G) from an elongated coil to a compact-globule state without an intermediate state.[30,31] The typical structure resulting from compaction is a toroidal structure accompanied by tight and regular packing of DNA segments (Figure 7H). In contrast, DNA compaction by **2** (Figure 7C) does pass through an intermediate state in which coil and compact parts coexist in a single DNA molecule (Figure 7B). Complex **2** gives compact DNA with an irregularly packed structure (Figure 7D). Furthermore, **2** induces complete DNA compaction at 0.02 mM, which is a surprisingly low concentration compared to that of spermidine (around 1 mM); the positive charge of spermidine (3+) is even higher than that of complex **2** (2+). In relation to this, it is known that divalent cationic compounds cause no apparent change in the higher-order structure of DNA at concentrations on the order of millimolar (Figure 7E), while high concentrations (above several tens of millimolar) of diaminoalkane (2+) or manganese (2+) can induce compaction or condensation of DNA.[32,33]

Therefore, these characteristics of **1** and **2** are markedly different from those of the usual divalent and trivalent cationic compounds, which suggests that interaction of platinum(II) complexes with DNA is not a simple electrostatic interaction, as discussed above. We previously reported that a change in the higher-order structure also occurs in the presence of cisplatin, an electrically neutral molecule.[34] Even with a higher concentration of cisplatin (0.1 mM) and a longer incubation time (24 h), T4 DNA did not reach a completely compacted state and mostly existed as a partial globule. Thus, the high antitumor efficacy of **2** may be attributable to the more efficient DNA compaction.

We will now briefly discuss the physicochemical aspects of the change in the higher-order structure of single long DNA molecules induced by **1** and **2**. It has been well established that a long DNA molecule exists as a semiflexible polymer; the contour length (or full length) is much larger than the

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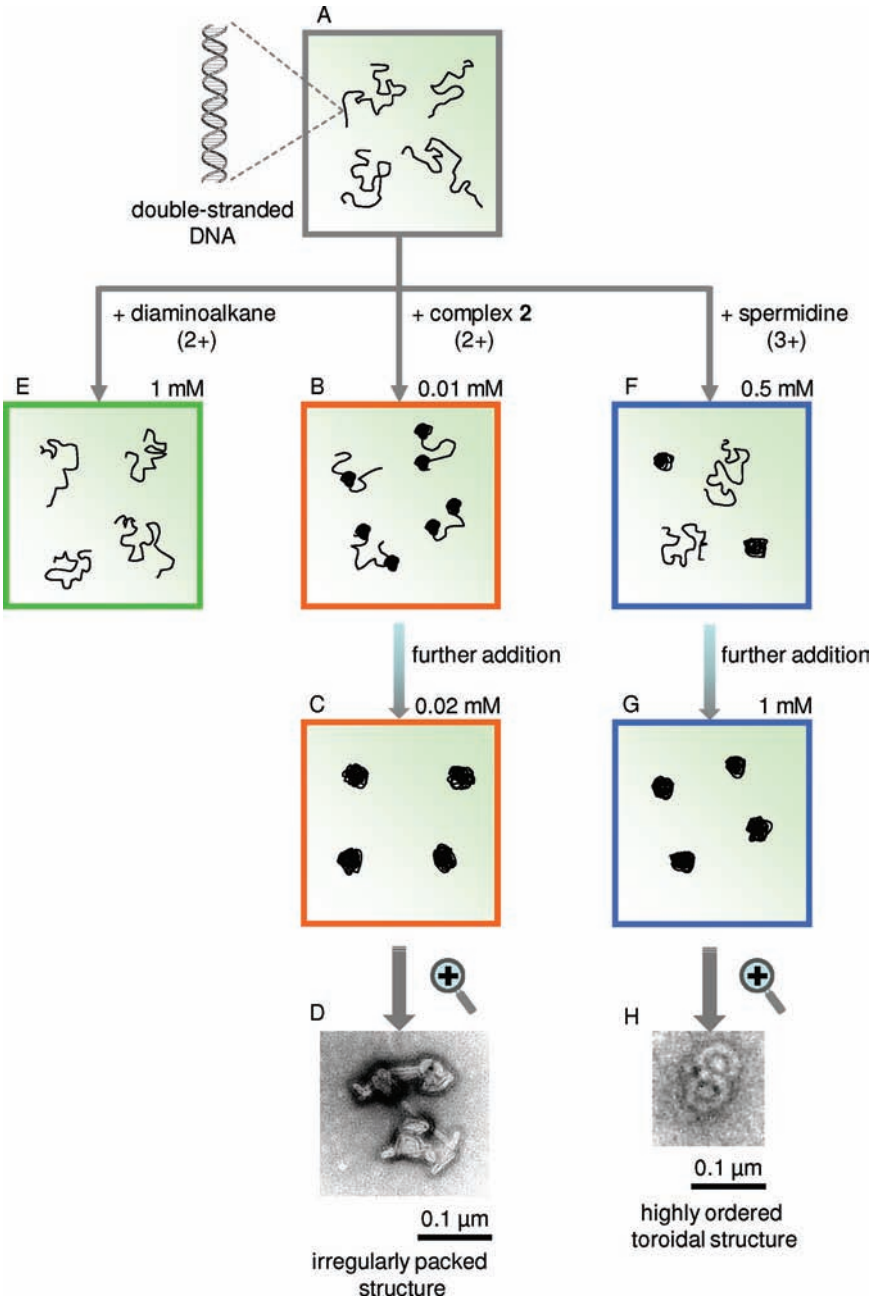

Figure 7. Schematic representation of the change in the conformation of a single DNA molecule induced by **2** (B and C) in comparison to those induced by divalent (E) and trivalent (F and G) cations. (D and H) TEM images of compacted DNA particles.

persistence length of DNA (ca. 50 nm, or 160 bp), and the persistence length is much larger than the diameter of double-stranded DNA (2 nm).[35] As an important property of long DNA above the size of several tens of kilo base pairs, individual DNA molecules undergo on/off switching, or a large discrete transition, between swollen coil and folded compact states, accompanied by a decrease of the size (radius of gyration and hydrodynamic radius) on the order of $^{1}/_{30}$–$^{1}/_{50}$. Such a change corresponds to a density difference on the order of 10^{4}–10^{5}. The contour length of T4 DNA that we used in the present study is 57 μm and thus fulfills the criterion as a semiflexible polymer. On the other hand, a short DNA oligomer with the same order of the persistence length behaves as a stiff rod. Such short DNA exhibits aggregation upon the addition of condensing agents without the occurrence of a folding transition.[14,36] Note that all genomic DNA molecules are long enough to be characterized as a semiflexible polymer. In addition to this semiflexible characteristic, we have to consider the properties of DNA as a highly negatively charged polymer; DNA segments usually repel each other in an aqueous environment. To overcome the repulsive electrostatic interaction between negatively charged segments of DNA, sufficient attraction should be imposed to achieve the condensation/compaction of DNA, as shown in the present experimental results. Sufficient attraction is only achieved when DNA segments exhibit parallel alignments mediated through the plural number of cross-linking formations between segments by the dinuclear platinum(II) complexes. In fact, the image in Figure 4 clearly shows the formation of parallel alignment on the compact structure of DNA. Interestingly, the significant

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change in the higher-order structure is accompanied by a change in the secondary structure as represented by CD measurements (Figure 6). Inspection of the differences between the changes in the higher-order and secondary structures indicates that the change in the secondary structure precedes compaction. Thus, the dinuclear platinum(II) complexes bind to DNA gradually with an *increase* in the *dose*. This may correspond to formation of the intermediate structure of long DNA, i.e., generation of the segregated, or partially compacted, conformation represented by the gray regions on the histogram in Figure 4.

In the present study, we studied the effect of platinum(II) complexes at 5−100 μM. As for the effective concentration of drugs with respect to living cells, we may consider the effect of crowding the intracellular environment. It is well-known that DNA facilitates the compact state in the coexistence of other macromolecules.[37] This crowding effect may promote the potentiality of the platinum(II) complexes to induce DNA compaction at relatively lower concentrations of the drugs in the intracellular environment. It remains an open problem how compaction of long DNA observed in vitro concerns the actual effect of the drugs on cancer cells; however, our results may suggest the possible action of an anticancer drug through modification of the higher-order structure on genomic DNA.

CONCLUSIONS

In the present study, we found that tetrazolato-bridged dinuclear platinum(II) complexes induced the irreversible compaction of large genomic DNA based on the results of single-molecule observation in aqueous solution, which was accompanied by a change in the secondary structure from the B to C form. Compound **2** was much more potent at inducing DNA compaction than **1**.

Azolato-bridged dinuclear platinum(II) complexes are known to interact with DNA in a mode that is different from that seen with cisplatin, suggesting that unique Pt−DNA interaction modes could lead to more effective antitumor activity toward platinum-refractory and chemotherapy-resistant tumors. Complex **2** showed highly efficient DNA compaction through a unique route. However, the search for coordination compounds that can compact DNA is still at an early stage. Cationic transition-metal complexes possess positive charges and molecular structures that can be modified with various ligands. Therefore, **2** may be a favorable foundation not only as anticancer drug candidates but also for the further development of efficient DNA compaction agents.

It has been reported that **2** exhibits higher antitumor efficacy than **1**.[9] The present results show the higher potency of the former on DNA compaction. This correspondence suggests that studies on the relationship between DNA compaction and the cytotoxic properties will need to be further developed.

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