Research paper

DNA compaction by mononuclear platinum cancer drug cisplatin and the trisplatinum anticancer agent BBR3464: Differences and similarities

T. Banerjeea, P. Dubeyb,1, R. Mukhopadhyayac,∗

a Department of Biological Chemistry, Indian Association for the Cultivation of Science, 2A&B, Raja S. C. Mullick Road, Kolkata 700032, India
b Department of Chemistry, Indian Institute of Technology, Kanpur 208016, India

c Corresponding author. Tel.: +91 33 2473 4071.
E-mail address: bcm@iacs.res.in (R. Mukhopadhyay).

1 Present address: Centre of Material Sciences, University of Allahabad, Allahabad 211002, India.

1. Introduction

Platinum compounds have been used as important chemotherapeutic agents for several decades [1–4]. Cis-diaminedichloroplatinum(II) or cisplatin (Fig. 1A), a mononuclear platinum compound, whose anticancer activity is thought to be associated to an ability of binding to DNA [5], is the first platinum-based cancer drug [6]. It is also one of the most widely used cancer drugs among all the platinum drugs. However, cisplatin has several drawbacks, e.g., toxicity problem and side effects, which limit its applicability to a great extent. Also, this drug can be used for not all, but some types, of cancer. So the alternatives have been sought for and several multinuclear platinum compounds have been developed so far [7,9]. BBR3464 is a trinuclear platinum compound, where two terminal trans-[PtCl(NH3)2] units are linked by a tetra amine [trans-Pt(NH3)2{H2N(CH2)6NH2}2]2+ unit (Fig. 1B) [10]. The two platinum ends can form co-ordinate covalent bonds with the DNA bases, preferentially the guanine residues [7]. Due to such binding, where both the platinum ends can be used, various types of cross-links, e.g., intra-strand cross-links, and 1,2-, 1,4- and 1,6-inter-strand cross-links [7,10], where the central tetra amine platinum unit is positioned at the minor groove of DNA [8,10], can be formed. BBR3464 is considered to be an important anticancer agent since it is expected to treat lung [11], pancreatic [12] and skin cancer [13], which are rarely treatable by current chemotherapy. It is also effective in cisplatin-resistant cancer cells [7,8]. Furthermore, it can function at 10 [7] to 1000 [8] times lower dose level compared to that applicable for cisplatin treatment.

The reasons behind the effectiveness of BBR3464 at a much lower dose limit compared to the typical cisplatin dosage values are not well-understood. Since it is increasingly being recognized that single molecule level experiments can offer new insights in understanding the behavior of biomolecules and the nature of...
biological events, high-resolution AFM, which is an established single molecule detection method, was applied in the present study. Though a number of reports that are based on molecularly resolved AFM imaging experiments, on understanding the effects of cisplatin on DNA molecules, have been made [14–17], only one report on the effects of BBR3464 treatment is available so far [18]. In this sole report, it has been shown that BBR3464 can induce drastic structural changes of DNA, in terms of overall compaction of the DNA molecules, at a molar ratio 0.01, which is 50–500 times lower than the molar ratio values relevant for cisplatin treatment [18]. However, any further details, especially information on the drug-induced local topological changes, are lacking. Therefore, in this study, we have performed experiments using uncoiled and nearly linear DNA molecules that can be generated by the molecular combing method [17], so that the regions of local distortion, e.g., loop structures, kinks etc., can be identified. We have studied the effects of both cisplatin and BBR3464 using the same DNA sequence and similar sample deposition method so that a comparison between the DNA compacting effects of cisplatin and BBR3464 can be made. Apart from applying the molecular combing method, a second method of deposition of the DNA-drug complexes onto 3-aminopropyltriethoxysilane (APTES) treated mica surface, was also adopted. The latter method, when modification is mild, allows one to observe the complexes in configurations that nearly represent the respective solution structures, and therefore helps one to obtain an idea about the overall structural/topological changes of the whole DNA molecule.

AFM is a well-established method for obtaining direct visual information on the structural aspects of various biological systems and events [19,20]. Recently, it has been shown that great details about the effects of drug-binding could be obtained from the molecularly resolved AFM images, in comparison to the conventional bulk phase studies, while the AFM data could also be correlated to the bulk phase information [18,21]. We will show about the effects of drug-binding could be obtained from the molecularly resolved AFM images, in comparison to the conventional bulk phase studies, while the AFM data could also be correlated to the bulk phase information [18,21]. We will show.

2. Materials and methods

2.1. Preparation of platinum compounds

BBR3464 was synthesized following the published literature [22]. Cisplatin was prepared according to the reported method [23,24]. Ultrapure water of resistivity 18.2 MΩcm (Millipore) was autoclaved and used for all sample preparation.

2.2. Preparation of DNA fragments

The DNA fragments (3650 bp) from pBR322 plasmid (Bangalore Genei, India) were prepared by carrying out restriction digestion of the plasmid with the restriction enzyme DraI (Bangalore Genei, India). The 3650 bp fragment was isolated from the other fragments by gel electrophoresis. Low-melting agarose (Bangalore Genei, India) gel (1%) was run at room temperature (23 ± 2 °C) in 0.5 × TBE (Tris-borate/EDTA) (Bangalore Genei, India) buffer at 30 V/cm for 3 h. Subsequent extraction of the fragment was performed using a gel purification kit (Qiagen). The DNA solution was eluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) and stored in −20 °C freezer in several stocks. The samples of DNA-drug complexes for AFM study were prepared with the fragments in 1 mM Tris–HCl, 0.1 mM EDTA, pH 7.4 buffer.

2.3. Preparation of cisplatin and BBR3464 solution

Both the platinum compounds (cisplatin and BBR3464) were first weighed using a 4-5-digit analytical balance. A known mass of each of the compounds was then dissolved in a known volume of autoclaved filtered Milli-Q water. The cisplatin solution and the BBR3464 solution were then kept undisturbed for 1 and 2 days, respectively, so that any undissolved particle could settle down. After 1 or 2 days, the supernatant solution was pipetted out as much as possible from the drug solution using an aspirapipette so that the volume of the supernatant solution was accurately known. Then the residue was dried completely and weighed. The weight of the dissolved part was equal to the total weight of platinum compound taken initially minus the weight of the residual part. The accurate volume of the dissolved part was known since the supernatant solution was pipetted out using an aspirapipette. So the concentration of the platinum compounds could be known from weight/volume calculation. The concentrations of the cisplatin and the BBR3464 solutions were found to be 326.63 ng/μl and 1052 ng/μl, respectively. For preparation of the DNA-drug complexes, these solutions were further diluted, up to a required level, with autoclaved filtered Milli-Q water.

2.4. Preparation of DNA-drug complexes

The DNA-drug complexes were prepared by mixing the DNA fragment solution of concentration 2 ng/μl and respective drug solutions at r$_1$ (ratio between free Pt complex and nucleotide phosphate) values of 1.5 (for cisplatin) and 0.03 (for BBR3464). The solutions were kept in dark at 37 °C for a total period of 48 h. An aliquot of 5 μl was taken out at specific time intervals (6 h, 12 h, 24 h and 48 h) and diluted to DNA concentration of 1 ng/μl for AFM imaging.

2.5. Preparation of sample for AFM experiments

Two different methods for attachment of DNA molecules onto mica surface have been employed.

(i) One freshly cleaved muscovite mica piece (ICR & Sons Pvt. Ltd., India) was positioned on an inclined plane (tilt angle 45°). Then a 10 μl droplet of 1 ng/μl DNA and 1 mM Ni$^{2+}$ was deposited on the top of the surface. After the droplet was slowly flown from the top to the bottom of the surface, the extra solution was gently removed with a micropipette. Then the mica foil was washed with 1 ml (4 × 250 μl) water [17,25]. By this method of attachment, the DNA molecules can get uncoiled and almost
linear on the surface, thereby revealing the nature and the positions of local distortions along the DNA contour.

(ii) One muscovite mica piece was freshly cleaved and kept in contact with an aqueous suspension of 3-aminopropyltriethoxysilane or APTES (Lancaster, UK) (water/APTES = 10,000:1 v/v) for 1 min. It was washed with 2 ml water, dried with a gentle stream of nitrogen gas and used immediately for sample deposition. APTES can generate positively charged surface, which is essential for immobilization of polyanionic DNA molecules. Brief modification time and use of highly diluted APTES solution should ensure mild modification of mica surface, which is necessary for minimizing the substrate effects on DNA solution conformations during adsorption. For preparation of samples with free DNA, 10 μl of the 3650 bp DNA solution in TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 7.4) of concentration 1 ng/μl was deposited onto AP-mica and kept for 20 min. The sample was then gently washed with 1 ml water, dried with a gentle stream of nitrogen gas and imaged immediately. For preparation of samples with DNA-drug complexes, similar methods of sample preparation as for free DNA were followed.

2.6. AFM data acquisition and analysis

All the AFM images were obtained in air, at 25 ± 1 °C and at about 45% humidity. The AFM experiments were performed using Agilent PicoLE equipment. The oscillatory motion of the cantilever was induced by applying an acoustic signal in the acoustic alternating current (AAC) mode. The cantilevers (μmasch) of spring constants 4.5–14 N/m were used within the frequency range 140–330 kHz. The tips were cleaned in a UV-ozone cleaner (Bioforce, NanoSciences) prior to imaging and cleanliness was checked from the relatively hysteresis free force–distance curve. The tip was engaged in the feedback loop at zero scan range condition to avoid tip contamination during the engage step. The amplitude set point was 80–90% of the free oscillation amplitude (7.5–8.0 V). Scan speed was typically 0.9–1.3 lines/s. All the images presented in this report are topographic images. Data analysis was carried out using the Picoscan 5.3.3 software. Minimum processing limited to leveling via plane correction (first to third order) was performed. Dimensional analyses of the free DNA molecules and the DNA-drug complexes were performed on 25–30 molecules/complexes each. For contour length measurement of free DNA molecules, DNA contours were manually traced using segmented lines. For width and height measurement of the DNA-drug complexes, the cross-sections were drawn along the longest continuous path over the central condensed region. The width value was obtained by measuring full width at half maximum of the cross-section profile, and the height value was obtained from the difference between the highest point in the cross-section and the average baseline of the cross-section.

2.7. Dynamic light scattering (DLS) experiments

Particle size measurements were performed by Dynamic Light Scattering (DLS) experiments using a Brookhaven instrument (BI 200SM Goniometer version 2.0). The data were recorded in TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 7.4) environment at 25 °C. A minimum of three measurements was performed on each sample. The autocorrelation curves were deconvoluted with either Cumulant, Exponential Sampling or Contin methods.

3. Results

In the present study, the DNA structural changes that are induced by cisplatin — a mononuclear platinum anticancer drug, and by BBR3464 — a trinuclear platinum anticancer agent, have been probed by high-resolution AFM. A fragment of pH322 plasmid DNA, which is sufficiently long (3650 bp in size) for making unambiguous detection of local distortions like loop structures, kinks etc. possible, was taken as an experimental model system. The closed circular DNA was not used, since the obvious presence of supercoiling in such a system could result in difficulties in clear discrimination between the loop structures that are formed due to drug treatment and the naturally occurring supercoiled regions of DNA. Complexation reaction between DNA and the platinum compounds were carried out in near-intracellular environment, i.e. at pH 7.4, in dark and at 37 °C. Low-chloride condition, as present within a cell, was maintained, so that the platinum compounds could remain in hydrolyzed condition and could form adducts with DNA. The drug:DNA molar ratio values were selected to be 1.5 for cisplatin and 0.03, which is 50 times less, for BBR3464.2 Drug treatment was carried out continuously till 48 h and aliquots were collected at the end of 6 h, 12 h, 24 h and finally 48 h, for the AFM studies. Two different methods of DNA immobilization were used. First, the usual deposition method using APTES treated mica (AP-mica) surface that allows the solution conformations of the DNA molecules to be nearly preserved on the substrate surface, was applied. Second, the molecular combing method [17] that results in uncoiling of the DNA molecules during adsorption on the substrate surface and therefore helps in revealing the points of local structural changes, was also applied. AFM imaging experiments were carried out in the intermittent contact mode and scan parameters were suitably adjusted to minimize tip-induced damage of the soft DNA molecules.

3.1. Compaction of the whole DNA molecule

The effects of both cisplatin and BBR3464 on DNA molecules were first studied using mildly modified AP-mica surface as the substrate. On such a surface, DNA molecules can be kinetically trapped [26], which implies that the DNA molecules are least mobile when adsorbed onto the substrate surface. It is therefore possible that the solution conformations of the DNA-drug complexes are kept nearly intact on the AP-mica surface. We did apply Mg2+ for DNA immobilization on mica surface since Mg2+ treatment results in weak attachment of the DNA molecules. As a consequence of such attachment, the DNA molecules can remain mobile and equilibrate on the surface [26], finally adopting 2D conformations that are formed under the influence of the substrate features. In Fig. 2A–F, are shown the single isolated free DNA molecules adsorbed onto AP-mica surface. Bends and coiled regions that are expected for a 3650 bp long dsDNA strand were observed in these images.

Cisplatin treatment of the DNA fragments resulted in formation of loosely compacted DNA-drug complexes, which displayed clear contacts between the dsDNA strands, within the first 6 h of incubation (Fig. 3A). The complexes exhibiting greater degree of

2 In one seminal study on BBR3464’s effects on DNA [7], which was based on biochemical assays, the BBR3464:DNA ratios from 0.01 to 0.05 were applied. Therefore, in the present study, we applied a ratio that falls within this range, so that the AFM data could be related to the information obtained from the earlier biochemical experiments. Also, in one of our earlier studies based on AFM imaging [18], we applied the ratio of 0.01, and we observed the DNA molecules became compacted upon BBR3464 treatment over a total period of 48 h. Therefore, in the present study, we kept the ratio near about this value and sought for details regarding the relevance of DNA compaction to the relatively stronger cytotoxic effects of BBR3464 compared to cisplatin. In order to develop an understanding of BBR3464’s cytotoxic effects at a lower dose limit than cisplatin’s, we compared the effect of BBR3464 on DNA molecular structure with that of cisplatin at 50 times lower concentration, and therefore, applied the cisplatin:DNA ratio of 1.5.
compaction, along with some molecules in relatively loosely compacted forms, were observed in the next 6 h of incubation (Fig. 3B). The degree of compaction increased and more tightly compacted complexes were observed as the incubation time period was increased to 24 h (Fig. 3CI and CII). Loosely compacted DNA molecules could however still be detected. After 48 h of incubation, a large number of compacted complexes possessing a highly condensed central region, which was often surrounded by loosely held DNA strands (see the magnified images shown in Fig. 4CIII and CIV). Loosely associated DNA fragments were still observed at this intermediate stage of incubation (Fig. 4CI, CII and CV). These complexes were observed in abundance and were quite large in size — almost twice compared to the complexes observed in case of cisplatin treatment (see Table 1). Uncompacted fragments that appeared like the untreated DNA molecules were observed for all the incubation periods, though with lesser frequency for longer incubation times.

3.2. Local compaction of DNA: formation of microloop and macroloop structures

In order to detect the local structural alterations that would otherwise be hidden inside the fully compacted form, the DNA molecules were uncoiled and made almost linear by the molecular combing method [17]. In Fig. 5A–F, are shown the uncoiled free DNA fragments adsorbed onto mica surface. The average contour length of these molecules was estimated to be 1060.49 nm (standard deviation: 152.7 nm) and the height value of the dsDNA strand was found to be 0.4 ± 0.2 nm. Considering the estimated contour length value, the helical rise was found to be 0.29 nm/bp, which is intermediate between the helical rise of the B- (0.34 nm/bp) and the A (0.24 nm/bp) form of DNA. It has been established earlier that direct information about the length of individual DNA molecules immobilized on a 2-dimensional surface can be obtained by AFM [27,28]. In fact, in one earlier AFM study on the structural effects of echinomycin on DNA, by Tseng et al. [29], the contour length of a 398 bp DNA fragment was found to be in between the length of

![Figure 2](image1.png)

**Fig. 2.** Free DNA molecules deposited onto AP-mica surface. Scale bar: 200 nm. Z-ranges in nm: (A) 0–0.8, (B) 0–1.0, (C) 0–0.8, (D) 0–1.0, (E) 0–0.8, (F) 0–0.8.

![Figure 3](image2.png)

**Fig. 3.** DNA-cisplatin complex (κ = 1.5) deposited onto AP-mica surface after incubation for different time periods (A) 6 h, (B) 12 h, (C) 24 h, (D) 48 h. Scale bar: 200 nm. Z-ranges in nm: (A) 0–0.7, (B) 0–1.1, (C) 0–0.9, (D) 0–0.9, (D) 0–2.8, (DII) 0–1.0.

A small number of tightly compacted complexes were also observed at this initial stage of drug treatment. The tightly compacted complexes were observed more frequently after 12 h (Fig. 4Bl and BII) and quite often after 24 h of treatment (Fig. 4CI–CIV). These complexes possessed a condensed central core, which was often surrounded by loosely held DNA strands (see the magnified images shown in Fig. 4CIII and CIV). Loosely associated DNA fragments were still observed at this intermediate stage of incubation (Fig. 4CI, CII and CV). The tightly compacted complexes, observed at the end of 48 h incubation, were highly condensed and took the appearance of compact globules (see Fig. 4D). These complexes were observed in abundance and were quite large in size — almost twice compared to the complexes observed in case of cisplatin treatment (see Table 1). Uncompacted fragments that appeared like the untreated DNA molecules were observed for all the incubation periods, though with lesser frequency for longer incubation times.
the A- and the B form of DNA, where the length was measured from the AFM images. In the present study, since we made a comparison between the effects of cisplatin and BBR3464 on DNA structure, keeping the dsDNA strand same in both the cases, the fact that whether the DNA molecules were intermediate between the A form and the B form should not make the comparative analysis different than that we present here.

Upon treatment with cisplatin, almost no change took place in the first 6 h of incubation (see Fig. 6AI) except for formation of a small number of microloops (Fig. 6AII–AIV). After 12 h of incubation, the microloops were frequently observed along with a small number of macroloops (Fig. 6BII–BIII). After 24 h of incubation, the extent of microloop formation increased noticeably (Fig. 6CII–CV), while macroloops were also observed, though only occasionally. Similar observations, as in case of 24 h of incubation, were made at the end of drug treatment, i.e., 48 h of incubation (Fig. 6DII–DV).

BBR3464 treatment of the DNA fragments resulted in formation of microloops (Fig. 7AI–AIV) as well as macroloops (Fig. 7AII–AV) within the first 6 h of incubation. DNA molecules having no such loop structures were also observed (Fig. 7AVI). With increase in incubation time period to 12 h, a number of macroloops were observed (Fig. 7BI–BIV) along with some microloops (Fig. 7BII and BIII). After 24 h, the observation was quite similar, i.e., both microloops and macroloops were observed (Fig. 7CII–CIII). In addition, intermolecular contacts resulting in macroloop-like structure could also be observed (Fig. 7CIV). At the end of 48 h of incubation, macroloops were observed most frequently (Fig. 7DII–DIV). Multiple macroloops within one molecule could be observed (Fig. 7DII) and even large aggregates of DNA molecules could be detected (Fig. 7DV). A small number of DNA molecules appearing as untreated molecules or at most with only one or two microloops could still be found at this final stage of drug treatment (Fig. 7DIV and DVI). It is important to note here that often the macroloop junctions (see Fig. 7AV, BII, BIII, CII, DI and DIII) appeared as an extended thick region of height value 0.7 ± 0.4 nm, which is almost two times the height value for one dsDNA strand. Such extended junction regions are shown highlighted by using white arrow marks in Fig. 7AV, BII, BIII, CII, DI and DIII. These junctions could have been formed due to bridging of two guanine-rich regions of dsDNA strand by BBR3464 molecules. Given the fact that there are two terminal platinum units in BBR3464 that are available for DNA cross-linking, and that these platinum units are separated by a linear flexible long backbone, it is suggested that such bridging action of BBR3464 is quite possible. There are

Table 1
Dimensional analyses of the compacted DNA-drug complexes formed at the end of drug treatment, i.e., 48 h. The complexes were imaged by AFM after deposition onto AP-mica surface.

<table>
<thead>
<tr>
<th>Type of complex</th>
<th>Width (nm)</th>
<th>Height (nm)</th>
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<tbody>
<tr>
<td>DNA-cisplatin</td>
<td>49.2 ± 24.7</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>DNA-BBR3464</td>
<td>95.5 ± 12.4</td>
<td>4.7 ± 1.4</td>
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Fig. 4. DNA-BBR3464 complexes (r1 = 0.03) deposited onto AP-mica surface after incubation for (A) 6 h, (BI) and (BII) 12 h, (CI)–(CV) 24 h, (D) 48 h. Scale bar for (A) to (CI), (D): 200 nm and for (CI)–(CV): 100 nm, Z-ranges in nm: (A) 0–0.8, (BI) 0–1.5, (BII) 0–2.1, (CI) 0–1.8, (CII) 0–1.5, (CIII) 0–1.6, (CV) 0–2.3, (CV) 0–1.2, (D) 0–1.6.

Fig. 5. Untreated DNA molecules in uncoiled forms on mica surface. Scale bar: 200 nm, Z-ranges in nm: (A) 0–1.3, (B) 0–1.0, (C) 0–1.0 (D) 0–1.1, (E) 0–1.1, (F) 0–1.1.
Fig. 6. Cisplatin-treated DNA molecules ($r_1 = 1.5$), in uncoiled forms, after incubation for (AI)–(AIV) 6 h, (BI)–(BIII) 12 h, (CI)–(CIV) 24 h, (DI)–(DV) 48 h. Some of the microloops are highlighted by white arrow marks in figures AIII, AV, BI, CI, DII and DIV. Scale bar: 200 nm, Z-ranges in nm: (AI) 0–0.8, (AII) 0–0.8, (AIII) 0–1.1, (AIV) 0–1.0, (BI) 0–1.0, (BII) 0–1.0, (BIII) 0–1.0, (CI) 0–1.1, (CII) 0–1.2, (CIII) 0–1.1, (CIV) 0–1.2, (DI) 0–0.6, (DII) 0–0.6, (DIII) 0–0.4, (DIV) 0–0.4, (DV) 0–0.4.

Fig. 7. BBR3464-treated DNA molecules ($r_1 = 0.03$), in uncoiled forms, after incubation for (AI)–(AVI) 6 h, (BI)–(BIV) 12 h, (CI)–(CIV) 24 h, (DI)–(DVI) 48 h. Some examples of the extended macroloop junction regions are highlighted with white arrow marks in figures AV, BII, BIII, CIII, DII and DIII. Scale bar: 200 nm, Z-ranges in nm: (AI) 0–1.0, (AII) 0–0.7, (AIII) 0–1.0, (AIV) 0–1.0, (AV) 0–0.7, (AVI) 0–1.0, (BI) 0–1.0, (BII) 0–1.1, (BIII) 0–1.1, (BIV) 0–1.0, (CI) 0–1.0, (CII) 0–1.0, (CIII) 0–1.0, (CIV) 0–1.0, (DI) 0–0.8, (DII) 0–0.6, (DIII) 0–1.5, (DIV) 0–1.0, (DV) 0–1.8, (DVI) 0–1.1.
a number of guanine-rich regions in the 3650 bp fragment that are distributed over the total length of the fragment. From the contour length values of the macroloops (30% within the range 430–450 nm, 30% within the range 240–270 nm, 30% within the range 60–100 nm and 10% of about 200 nm length) and the contour length values of the two extensions hanging outside the loop (40% nearly equal, and the rest of various ratios like 1:2, 1:3, 3:4, 3:5 and 1:12), it can be concluded that the observed macroloop formation could have involved a number of combinations of the guanine-rich regions that are located at not in specific two or three, but a greater number of positions. The probable guanine-rich regions as identified on the basis of this analysis are shown in Fig. 9. The bridging points approximately correlate to these positions of the guanine-rich regions along the DNA strand. For example, bridging between the regions ‘133–186’ and ‘422–708’, which correlates to the loops of length of about 100 nm (i.e., of size of about 295 bp); bridging between the regions ‘1418–1514’ and ‘2708–2789’, which correlates to the loops of length of about 445 nm (i.e., of size of about 1310 bp); and bridging between the regions ‘2121–2209’ and ‘2387–2467’, which correlates to the loops of length of about 65 nm (i.e., of size of about 190 bp).

In order to confirm that cisplatin could not induce DNA structural changes that are as drastic as that induced by BBR3464 at the r value of 0.03, AFM experiments were performed on cisplatin-treated DNA molecules, in uncoiled condition, at r 0.03. Very little changes, except for a small number of microloops, could be occasionally detected (Fig. 8A), and the molecules appeared mostly like the free uncompacted DNA molecules (Fig. 8B–F).

3.3. Control experiments

For the control experiments, solutions each for cisplatin, BBR3464 and TE buffer were incubated at 37 °C in dark for 48 h, then deposited onto mica surface and imaged immediately. In the AFM images, no globular or irregular-shaped features that resembled the DNA-drug complexes could be detected (not shown). Only some particles of very small sizes were observed. Therefore, it can be confirmed that the compacted DNA structures were generated due to binding of the platinum compounds.

3.4. Dynamic light scattering (DLS) observation

For obtaining an idea about the DNA compaction/self-association behavior in bulk solution medium, DLS experiments were performed on cisplatin-treated DNA and BBR3464-treated DNA, at r values 1.5 and 0.03, respectively, for the incubation time periods 6 h, 24 h and 48 h. In case of cisplatin treatment, the effective diameter values of the DNA-drug complexes kept reducing with continued drug treatment over the total incubation period of 48 h (see Table 2). This observation is consistent with the AFM finding that the DNA molecules got compacted upon cisplatin treatment (Fig. 3). However, for BBR3464 treatment, the effective diameter values exhibited an increasing trend (see Table 2), which indicates the possibility of an event like intermolecular aggregation that could overcome the effects of compaction.

4. Discussion

In the present work, it is revealed from the high-resolution AFM imaging experiments that both cisplatin and BBR3464 can compact DNA molecules to a noticeable extent within the total time period of 48 h (see Figs. 3 and 4). Distinct differences were however also observed in the compacting behavior of these two platinum compounds. For example, the extent of compaction within a certain time period could be different – first, after initial 6 h of drug treatment, closely compacted complexes could be observed for BBR3464 treatment (Fig. 4A), but not cisplatin treatment (Fig. 3A); second, at the end of 48 h incubation, completely compacted complexes could be observed in case of BBR3464 treatment (Fig. 4D), but not cisplatin treatment (Fig. 3DII and DIII). An interesting point to note here is that macroloops were observed to form in considerably high number after the first 6 h incubation in case of BBR3464 treatment (see Fig. 7AII–AV), but not cisplatin treatment (Fig. 6AII–AIV). In fact, at any stage of incubation, macroloops were observed more frequently for BBR3464 treatment compared to the treatment with cisplatin. Since a large portion of DNA can be brought close together via macroloop formation, BBR3464’s greater ability of macroloop formation could have contributed to the greater degree of compaction (see Fig. 4A and D) induced by BBR3464 compared to cisplatin.

Since cisplatin treatment of DNA produced only a small number of macroloops, but a large number of microloops, DNA compaction as observed in case of cisplatin treatment could be associated more to the formation of microloops than the macroloops. Recently, Hou et al. have also shown that cisplatin can induce microloop formation at a number of places along the total length of a DNA molecule [17]. Formation of microloops indicates cisplatin’s ability of DNA cross-linking over a much longer range than the range relevant for 1.2 GG intra strand cross-linking, which is thought to be the primary binding mode of cisplatin [30]. Given the fact that cisplatin is a mononuclear compound and does not possess a long linear

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**Table 2**

The effective diameter values of the DNA-drug complexes for different incubation time periods as obtained from dynamic light scattering experiments.

<table>
<thead>
<tr>
<th>Type of complexes</th>
<th>Effective diameter (nm)</th>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>DNA-cisplatin</td>
<td>357.5</td>
</tr>
<tr>
<td>DNA-BBR3464</td>
<td>351.6</td>
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</table>
the long-range cross-links can be generated only when the long and conformationally flexible structure of BBR3464 would assist the free platinum terminal to access some of the DNA binding sites that are situated not very close to the bound platinum end.

There are a number of reports on the nature of cisplatin binding to DNA that describe cisplatin as a groove-binder [31–33]. BBR3464’s action as a groove-binder has also been clearly stated in several reports [8,10]. Intercalative binding of neither cisplatin nor BBR3464 has been suggested by any group so far. In our studies also, no clear evidence of intercalative binding has been obtained. Jon-pairing with phosphate is unlikely to be a major factor, since the degree of DNA compaction was observed to be considerably higher in case of BBR3464 treatment compared to cisplatin treatment, in spite of the fact that cisplatin was applied at a concentration 50 times higher than that of BBR3464.

Though it has been reported by Yotsuyanagi et al. that cisplatin can hydrolyze in a very complicated manner [34], it is generally accepted that upon hydrolysis the neutral species becomes a positively charged chloro-aqua and a di-aqua complex that can bind to DNA and form a mono-adduct and a di-adduct, respectively. The observation of microloop formation by cisplatin treatment indicates long-range DNA cross-linking via di-adduct formation [17]. The hydrolysis profile of BBR3464 has been reported by Davies et al. [35], where it has been shown that BBR3464 hydrolyzes much faster than cisplatin. As a result of hydrolysis of BBR3464, a chloro-aqua and a di-aqua complex can be generated, that can form a mono-adduct and a di-adduct, respectively, with DNA. Generation of microloops and macroloops by BBR3464 treatment indicates long-range DNA cross-linking through di-adduct formation. However, it has also been clearly stated by Wheate et al. [8] that though the hydrolysis rates of BBR3464 and cisplatin are different, this difference is not enough to explain the enormous difference in their cytotoxic behavior. It is now generally believed that the highly cytotoxic effects of the multinuclear platinum complexes is due to the novel type of adducts that they form with DNA.

One obvious effect of the presence of two terminal platinum units in BBR3464 could be the generation of intermolecular cross-links and formation of DNA intermolecular aggregates. In the present AFM study on DNA-BBR3464 complexes, large DNA aggregates could occasionally be observed during all the incubation stages. Indication of intermolecular DNA aggregation could be found also from an increase of the effective diameter value as obtained from the DLS experiments. Since evidence of DNA compaction could be obtained from the bulk solution phase circular dichroism and UV–visible experiments, as reported earlier [18], it must be that the effects of aggregation overcame the effects of compaction in the DLS experiments. In DLS, since one obtains the average size of all the molecules/complexes present in the bulk medium rather than the size of individual molecules/complexes, the effective diameter values must have reflected an average picture of all the different types of complexes — therefore taking into account the aggregated complexes. The AFM experiments can however be performed at the single molecule/single complex level and therefore the compacted non-aggregated complexes could be clearly detected in all the AFM experiments.

An ability of long-range cross-linking, in terms of microloop and macroloop formation by both the mononuclear cisplatin and the trinuclear BBR3464, is clearly elicted from the AFM experiments. BBR3464 could produce macroloops much more frequently than cisplatin could, and since macroloop formation means a greater length of DNA getting cross-linked compared to the length covered by the microloops, an advantage of BBR3464 clearly exists over cisplatin as far as loop-mediated compaction of DNA molecules is concerned. The exact relation between this advantage of BBR3464 and its cell-damaging ability at much lower molar ratio compared to that relevant for cisplatin treatment is not clear at the moment. This is because the exact role of the loop structures in inducing cytotoxic effects is yet to be found out. There remain questions — for example, how the binding of high mobility group (HMG) proteins [32] can be influenced by the presence of microloops and macroloops. However, a correlation between such loop structures and the cytotoxic effects could be present since the junction regions that are formed as a direct consequence of DNA cross-linking, would inhibit strand separation of dsDNA, which is an essential step in DNA replication. Such inhibition may produce more drastic effects in case of macroloops than the microloops, since a greater portion of DNA would remain inaccessible to the DNA unzipping event in case of macroloops. Compaction of the whole DNA molecule could also prevent access of the complete DNA sequence to the enzyme molecules that are relevant in DNA transcription.

5. Conclusion

In summary, we have shown that both the platinum compounds mononuclear cisplatin and trinuclear BBR3464 could compact dsDNA molecules, though the degree of compaction by BBR3464 was more, even for a molar ratio, which is 50 times lower than that relevant for cisplatin treatment. Differences in the nature and extent of loop formation could be identified from the molecularly resolved AFM topographs. Though the exact role of the local structural changes in terms of loop formation in inducing cytotoxic effects is yet to be revealed, the present study offers important details regarding the DNA structural alterations at the molecular level. Such changes are found to be more drastic in case of BBR3464 treatment, compared to cisplatin treatment even at a 50 times lower molar ratio, and therefore could possibly be correlative to BBR3464’s effectiveness at a lower dose limit compared to that relevant for cisplatin treatment.

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