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ARTICLE

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Studying the effect of a charged surface on the interaction of bleomycin with DNA using an atomic force microscope

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Abstract The cleavage of DNA caused by the antitumoral drug bleomycin has been investigated using atomic force microscopy (AFM). This work deals with the effect that adsorbing DNA onto a positively- or negatively-charged surface has on the double-strand cleavage of DNA by Fe(III)/bleomycin. Quantitative analysis of the number of breaks per DNA molecule, in bulk and at the surface of the mica substrate, has been performed by analyzing AFM images. It turns out that the cleavage of DNA is strongly inhibited by a positively-charged surface. Our experiments can be interpreted using a simple electrostatic model. This paper is a first step in the study of DNA accessibility to ligand such as bleomycin, using AFM in liquids.

Keywords Bleomycin · DNA cleavage · Atomic force microscope · Surface

Introduction

Bleomycin (Blm) is a metal-binding glycopeptide antibiotic used in anticancer chemotherapy. This drug binds to and cleaves DNA in a reaction which depends on the presence of ferrous ions and oxygen (Abraham et al. 1999; Burger 1998; Claussen and Long 1999; Petering et al. 1990; Povirk and Goldberg 1983). Blm induces both double- and single-strand breaks in DNA, and cleavage is reported to occur preferentially at the dinucleotide sequences 5′-GC-3′ and 5′-GT-3′ (D'Andrea and Haseltine 1978; Takeshita et al. 1978). Double-strand breaks probably occur during a single bleomycin binding event less frequently than single-strand breaks (Steighner and Povirk 1990). The cleavage reaction in cellular and in isolated DNA probably involves chelation of the ferrous ion by the drug, followed by binding of the Fe(III)-bleomycin or Fe(II)-bleomycin complex to DNA.

Studying the influence of the adsorption of DNA on the bleomycin activity may provide qualitative information useful in understanding the activity of bleomycin towards DNA immobilized on a highly-charged surface. In this paper we study the action of bleomycin on DNA molecules adsorbed on a mica surface using an atomic force microscope (AFM). AFM has been used to image DNA-drugs complexes (Berge et al. 2002; Nagami et al. 2002; Pope et al. 2000; Utsuno et al. 2001, 2002) and DNA-protein complexes on ultra-flat surfaces (Allison et al. 1996; Cary et al. 1997; Guthold et al. 1994; Lyubchenko et al. 1995; van Noort et al. 1998). Prior to imaging, it is necessary to adsorb the DNA molecules onto an atomically flat surface. This requirement is generally obtained by using a mica surface and a buffer containing divalent cations. The divalent cations partially neutralize the negatively-charged DNA backbone (Rouzina and Bloomfield 1996a) and the mica surface (Pashley and Israelachvili 1984). Correlations between the DNA and mica counterion clouds arise and generate a net attractive force that leads to adsorption of the DNA molecules onto mica (Pastré et al. 2003). This mechanism is very similar to the one for DNA condensation induced by multivalent cations (Rouzina and Bloomfield 1996b). AFM techniques present two main advantages for studying DNA/drug interactions. First, few biological materials are required (nanomolar DNA molecule concentrations are sufficient to perform DNA imaging). Second, it allows us to study the influence of

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the surface substrate on the formation of the drug/DNA complexes. In addition, drugs are generally small ligands that do not interact very strongly with a charged surface, unlike proteins, which are large and highly polarizable. We report the first AFM study of the action of bleomycin on naked DNA molecules adsorbed onto a surface.

This study focuses on bleomycin-induced DNA double-strand cleavage, which can be easily detected by AFM. A method is developed to measure the number of DNA double-strand breaks per molecule by assuming that the distribution of double-strand breaks among DNA molecules follows the Poisson distribution (Povirk and Houlgrave 1988). We show that bleomycin molecules, which are also electrostatically adsorbed onto the mica surface, are responsible for the DNA breaks in adsorbed molecules. A simple model is developed to explain the electrostatic influence of the surface. In addition, the surface potential of the mica surface can be modified by pre-treating the mica surface with Ni^{2+} ions (Piétrement et al. 2003). The point of this is to observe whether the non-specific electrostatic interactions between mica, DNA and bleomycin molecules can modulate the double-strand cleavage of DNA molecules adsorbed onto a surface.

Materials and methods

Bleomycin and DNA plasmid

Bleomycin sulfate (Blenoxane) was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Fe(III)-bleomycin was prepared by first dissolving ferric ammonium sulfate dodecahydrate in water and then immediately adding a 10% molar excess of iron to bleomycin (Li et al. 2002). This 1 mM Fe(III)-bleomycin solution was then kept at −20 °C.

pUC19 plasmid DNA was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Circular DNA molecules allow us to determine the DNA double-strand breakage more easily.

AFM experiments

These experiments were carried out in air using a Multimode AFM Nanoscope IIIa system (Digital Instrument/Veeco, Santa Barbara, CA, USA) in tapping-mode. We used OTESPA cantilevers (Digital Instruments/Veeco, Santa Barbara, CA, USA); the scan frequency was 1 Hz and the modulation amplitude was a few nanometers.

A droplet of DNA solution was deposited onto the mica surface as described below, according to whether the DNA was cleaved in a buffer solution or on a mica surface. All the samples were rinsed with 2–3 drops of 0.02% (w/v) aqueous uranyl acetate in order to fix the DNA molecules in their conformations (Revet and Fourcade 1998), and they were blotted with filtered paper.

Cleavage of DNA adsorbed onto mica by bleomycin

A 4 µl droplet of 2.5 µg/ml DNA diluted in a buffer solution (10 mM HEPES, 5 mM $MgCl_2$, pH 7.5) was deposited onto a mica substrate (pre-treated or not) for 2 min. Then 16 µl buffer solution containing Fe(III)-bleomycin at a specified concentration (with/without 100 µM ascorbate) was added. Bleomycin was allowed to interact for 4 min and then the sample was rinsed and dried as described above. A dilute DNA solution and a short deposition time are both required to limit the number of adsorbed DNA molecules. We have measured the density of DNA molecules adsorbed onto mica before and after incubation in buffer containing bleomycin. These conditions ensure that more than 90% of the DNA molecules observed on the surface by AFM were adsorbed onto the mica surface prior to the addition of the buffer containing bleomycin.

Cleavage of DNA in solution by bleomycin

A 4 µl droplet of 2.5 µg/ml DNA solution was added to 16 µl buffer solution containing Fe(III)-bleomycin with or without 100 µM ascorbate. Bleomycin interacts with DNA in 4 minutes. The reaction was stopped by adding 5 µl of 50 mM EDTA solution. The reaction solution was then deposited on a mica surface by adding $MgCl_2$ for AFM observation, rinsed with aqueous uranyl acetate, and dried.

Results and discussion

AFM measurements of the DNA double-strand breaks

Several techniques have been used previously to study DNA cleavage by bleomycin: gel electrophoresis (Lloyd et al. 1978), viscosity measurements (Povirk and Houlgrave 1988), and fluorescence (Biggins et al. 2000). Microscopy techniques have been used less frequently, which is in part due to the time consuming analysis of the DNA molecules. Lloyd et al. (1979) used electron microscopy to study the intermolecular crosslinking that could be produced by bleomycin on circular DNA. Comparisons between electrophoresis analysis and electron microscopy revealed that images of DNA molecules provide qualitative results as well. Therefore we have focused this study on double-strand breakage, which is easier to quantify by AFM image analysis.

By assuming that the distribution of double-strand breaks among individual molecules follows the Poisson distribution (Povirk and Houlgrave 1988), the probability $f_{n,i}$ that a DNA molecule will have exactly i breaks is equal to:

$$f_{n,i} = \mathrm{e}^{-n}\frac{n^i}{i!}, \tag{1}$$

with n being the mean number of double-strand breaks per molecule.

Figure 1 shows images of pUC19 plasmid DNA molecules adsorbed onto a mica surface and cleaved by 0.3 μM Fe(III)-bleomycin after 4 minutes incubation. Figure 2 shows the distribution in the lengths of the DNA molecules for $n = 1.65$. We can see that the experimental results seem to match the distribution in the DNA segment lengths (obtained numerically by assuming a Poisson distribution of the DNA breaks) pretty well:

- For a low level of DNA breaks ($n < 1$), n is given by the ratio between the number of linear DNA molecules with one double-strand break and the number of circular DNA molecules: $n = f_{n,1}/f_{n,0}$.
- In the intermediate range ($1 < n < 3$), the small linear DNA molecules should also be considered in order to improve the reliability of the method. Therefore, we calculate the total number of DNA molecules, and the ratio R of the number of circular DNA to the number of linear DNA molecules is given by:

$$R = \frac{f_{n,0}}{\sum_i i f_{n,i}} = \frac{\mathrm{e}^{-n}}{\sum_{i=1,2\ldots} \mathrm{e}^{-n}\frac{n^i}{(i-1)!}}. \tag{2}$$

This equation is solved numerically so that n is obtained from the experimentally-estimated value of R derived from AFM image analysis.

- For highly-damaged DNA ($n > 3$), we measure the lengths of at least 200 molecules. Then we determine the number of double-strand breaks n which best fits the theoretical DNA length distribution obtained by using the Poisson distribution.

Interaction between bleomycin and DNA molecules adsorbed onto a surface

The charged mica surface can modify the DNA/ligand interaction via several mechanisms. First, the surface can interact directly with the bleomycin molecule (only non-specific electrostatic interactions are considered here). Second, the surface can interact indirectly with DNA since its mobility is restrained by adsorption, which could affect the DNA/bleomycin interaction.

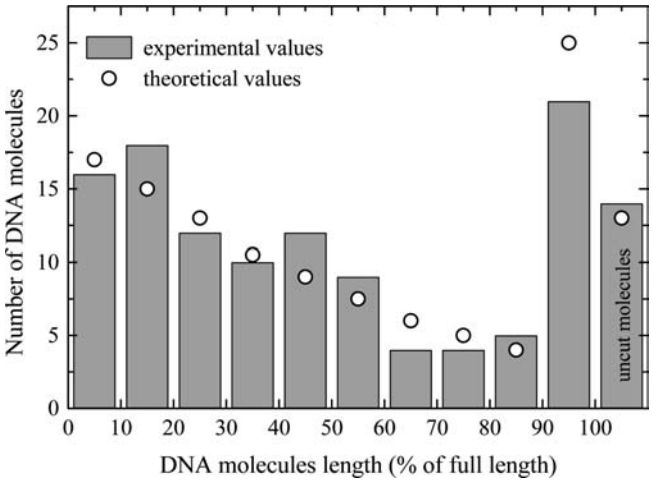

Fig. 2 Distribution of the DNA segment lengths after interaction of pUC19 circular DNA with 300 nM Fe(III)-bleomycin solution on a mica surface. The distribution of DNA segment lengths agrees pretty well with the simulated values, which attests to the validity of the Poisson distribution. To ensure the precision of the method, more than 150 molecules should be analyzed

Let us start by considering an electrostatic interaction between a charged ligand and a charged surface. On a highly-charged surface the counterions tend to form a thin condensed layer. Using the non-linear Poisson-Boltzmann equation, the decay in the counterion concentration is given by (Rouzina and Bloomfield 1996b):

$$n(d) = \frac{ns}{(1 + (d/2\lambda_z))^2} + n_\mathrm{b}, \tag{3}$$

where d is the distance from the surface, n_b is the bulk concentration of the counterions species, ns is the surface concentration of the mica counterion (ns is between 1 and 6 M for a mica surface):

$$\lambda_z = \frac{e}{4\pi\sigma l_\mathrm{B} z} \tag{4a}$$

and λ_z is the thickness of the counterion layer:

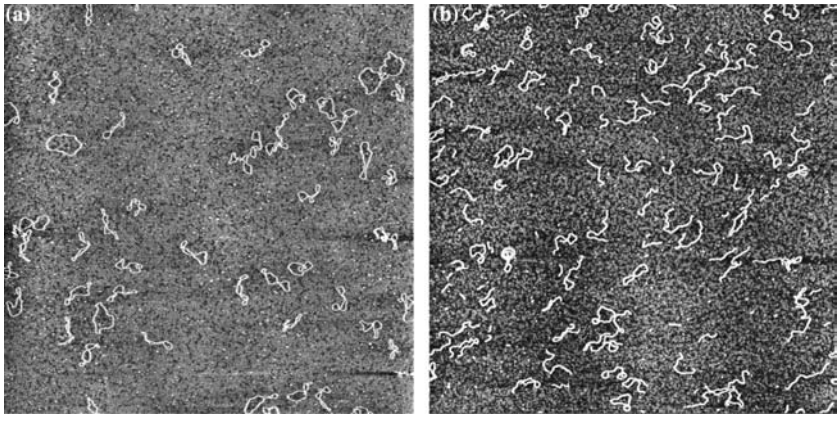

Fig. 1 **a** AFM image of untreated pUC19 circular DNA. Most of the molecules adopt a supercoiled conformation. **b** AFM image of adsorbed pUC19 circular DNA molecules after reacting for 4 min with 300 nM Fe(III)-bleomycin solution on a mica surface. The nanoscale resolution of the AFM allows the precise classification of each of the DNA molecules as linear or circular. Scan area: 5×5 μm^2

$$ns = 2\pi(\sigma/e)^2 l_B, \tag{4b}$$

where e is the electron charge, σ is the surface charge density, l_B is the Bjerrum length and z is the counterion valency.

Mg^{2+} and Hepes ions compete for surface neutralization. However, the Hepes ions are poorer competitors than the Mg^{2+} ions because of their size (Rouzina and Bloomfield 1996b), so we can only consider the Mg^{2+} counterions for the calculations. Figure 3 shows the decay of $n(d)$ for various $MgCl_2$ concentrations. The surface concentration of divalent counterions is much higher near the surface because of the condensation phenomenon. In addition, it is easy to see that the surface concentration is only weakly dependent on the bulk concentration for $d < 1$ nm. Theoretical calculations show that the DNA counterions should be located at a distance less than $l_B(\cong 0.7$ nm) from the surface in order to allow the adsorption of DNA (Pastré et al. 2003). Therefore, most of the adsorbed DNA molecules are embedded in the counterion layer in which the concentration of Mg^{2+} ions is weakly dependent on the buffer composition. This effect will be used to control whether the reaction between bleomycin and DNA molecules adsorbed onto mica really takes place on the surface or not, as described below.

Let us estimate the bleomycin concentration near the surface. The bleomycin carries a positive charge via the C-terminal cationic group of bleomycin A_2 that is involved in the DNA electrostatic attraction between DNA and bleomycin (Sakai et al. 1983). However, other bleomycin domains can be electrostatically active, like the DNA binding domain. For such a large ligand, an effective charge z_{eff} can be introduced (Rouzina and Bloomfield 1996b). The surface concentration of bleomycin ns_{blm} is then:

$$ns_{blm} = \frac{[Blm]}{[Mg^{2+}]^{(z_{eff}/2)}} ns^{(z_{eff}/2)}. \tag{5}$$

We can assume that $ns_{blm} \ll ns$ because the bleomycin concentration (< 1 μM) used to cleave DNA is lower than the $MgCl_2$ concentration (5 mM) necessary to adsorb DNA molecules onto a mica surface. This equation indicates that the concentration of bleomycin on the mica surface is larger than the concentration of bleomycin in solution. However, the surface concentration of divalent counterions is also very high, which can affect the DNA cleavage.

The mica surface charge can be modified by adding Ni^{2+} ions to the solution. Ni^{2+} ions are highly reactive with the mica, like other transition metal cations. In particular, Ni^{2+} ions are able to form $(Ni–OH)^+$ hydroxyl complexes (Gier and Johns 2000; Koppelman and Dillard 1977) thanks to the high ionic potential of Ni^{2+}. The strong adsorption of Ni^{2+} ions during pretreatment neutralizes the mica surface if most of the potassium ions are exchanged with the Ni^{2+} ions. The surface charge of the mica can therefore be neutralized or partly reversed by the strongly-adsorbed cations. This mechanism could be exploited to study the electrostatic interaction between the mica surface and the bleomycin. Indeed, a positively-charged surface could repel a positively-charged ligand such as bleomycin.

For all of the figures presented below, we measured the number of DNA breaks per molecule by analyzing at least 200 molecules (see "Materials and methods" section). These experiments were performed using the same buffer and with the same muscovite mica to avoid any additional errors.

Figure 4 represents the number of DNA breaks versus the magnesium concentration in the bulk and on the

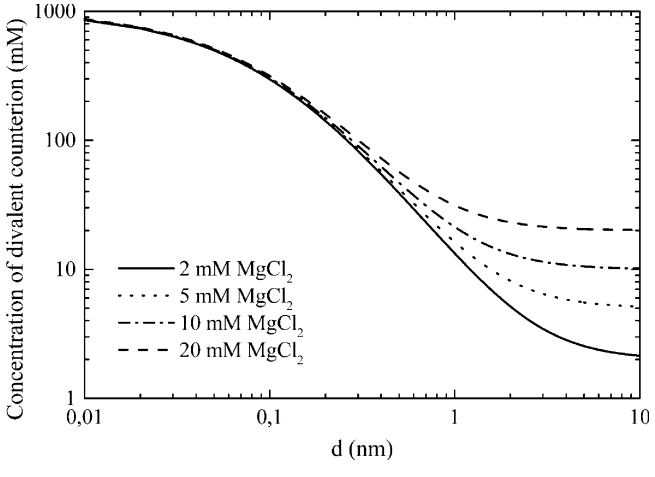

Fig. 3 Variation of the theoretical surface concentration of divalent counterions on a mica surface versus the distance d from the mica. Experiments were performed with several $MgCl_2$ concentrations (2, 5, 10, and 20 mM) and with $ns = 1$ M. For $d < 1$ nm, the surface counterion concentration is weakly dependent on the bulk concentration of the counterion, whereas for $d > 4$ nm it is similar to the bulk value

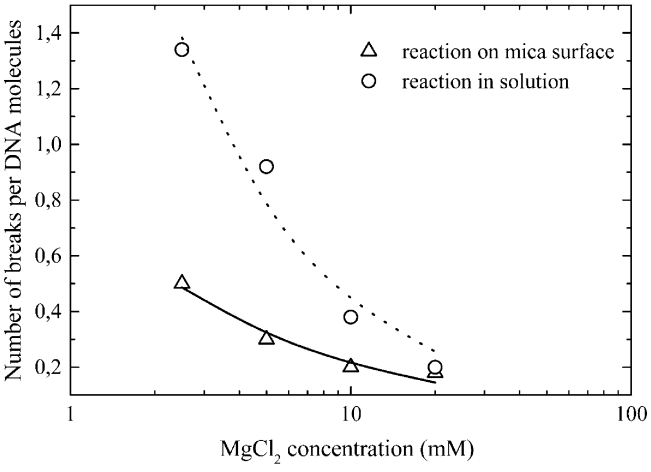

Fig. 4 Number of DNA breaks per molecule versus $MgCl_2$ concentration in bulk and on a mica surface, with [Fe-(III)BLM] = 30 nM and [Asc] = 100 μM. The experimental results are fitted by a polynomial function of the $MgCl_2$ concentration. This comes from Eq. (5) $\frac{z_{eff}}{2} = 0.55$. Inhibition of DNA cleavage by magnesium ions is less marked for adsorbed DNA molecules. This indicates that the DNA cleavage takes place in the electrical layer near the surface, as predicted by the model

surface. In bulk (reaction in solution), the magnesium is known to inhibit the cleavage of DNA by bleomycin (Chien et al. 1977; Takeshita et al. 1976). The plot of DNA breaks per molecule versus $MgCl_2$ concentration ($[MgCl_2]$) can be fitted by a polynomial function, which indicates that the number of DNA breaks per molecule is proportional to $[MgCl_2]^{-0.92}$. On the surface (when bleomycin interacts with adsorbed DNA molecules), the dependency of the cleavage of DNA by Fe(III)-bleomycin on the magnesium concentration is weaker than in the bulk ($[MgCl_2]^{-0.55}$). This result agrees with the electrostatic model, which predicts that DNA molecules adsorbed on a mica surface are located in the condensed counterion layer. As the concentration of magnesium condensed at the surface is weakly dependent on the bulk magnesium concentration, the DNA cleavage is weakly dependent on the bulk magnesium concentration. Using Eq. (3) and assuming that the number of DNA breaks is directly proportional to the bleomycin concentration at the surface ns_{blm}, we obtain the bleomycin effective charge $z_{eff} \approx 1.1$. This value is pretty accurate, since the bleomycin C-terminal cationic group carries only a single positive charge. More detailed calculations performed for the adsorption of DNA into netrospin and Hoechst 33258 show that the calculated values for these ligands are a little larger than their valencies due to the sizes and flexibilities of the ligands (Rouzina and Bloomfield 1996b). Equation (5) can provide a qualitative estimation of the Fe(III)-bleomycin concentration on the surface. When $[MgCl_2] = 5$ mM and [Fe(III)-bleomycin] = 30 nM, it turns outs that the surface concentration of the Fe(III)–bleomycin complex is about 0.5 μM, which is more than one order of magnitude larger than the bulk concentration. Moreover, bleomycin molecules adsorbed onto the mica surface prior to DNA deposition are able to damage DNA (data not shown), which agrees with the electrostatic adsorption hypothesis. We can therefore assume that part of the DNA damage results from bleomycin molecules adsorbed onto the surface.

For a more precise study of the effect of the surface, we have measured the number of DNA breaks per molecule versus the bleomycin concentration for bulk (Fig. 5) and adsorbed (Fig. 6) molecules, either with or without 100 μM ascorbate. Firstly, note that the number of DNA breaks in bulk is linearly dependent on the bleomycin concentration, and it is considerably enhanced by ascorbate addition, as expected for low bleomycin concentrations (Chien et al. 1977; Li et al. 2001). For DNA molecules adsorbed on the mica surface, the number of DNA breaks is not linearly dependent on the bleomycin concentration. These results indicate that the DNA/bleomycin interaction strongly depends on the mica surface.

In the absence of ascorbate, for a wide range of bleomycin concentrations, DNA molecules are damaged more on the mica surface than in bulk. This is in agreement with the electrostatic attraction of the bleomycin molecules towards the surface (Li et al. 2001). We

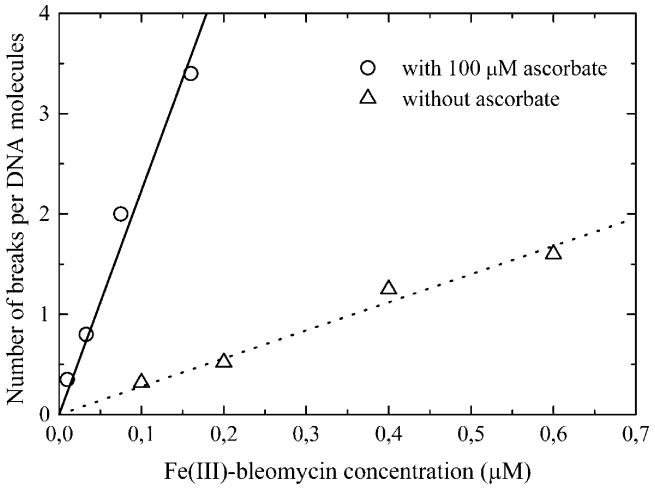

Fig. 5 Plot of the number of DNA breaks per molecule versus Fe(III)-bleomycin concentration for reactions in bulk. The measured values are obtained by analyzing AFM images of DNA molecules treated by Fe(III)-bleomycin at various concentrations with and without 100 μM ascorbate. The number of DNA breaks is linearly dependent on the bleomycin concentration. The presence of ascorbate leads to a significant amplification of the cleavage efficiency

may assume that the high concentration of bleomycin on the surface could favor electron reduction via a dioxygene-bridged dimer of Fe(III)-bleomycin (Petering et al. 1990).

In the presence of ascorbate, the enhancement of the cleavage of DNA is not as efficient as in bulk (see Fig. 5). Generally, the addition of excess ascorbate increases the potential of a bleomycin molecule to cleave

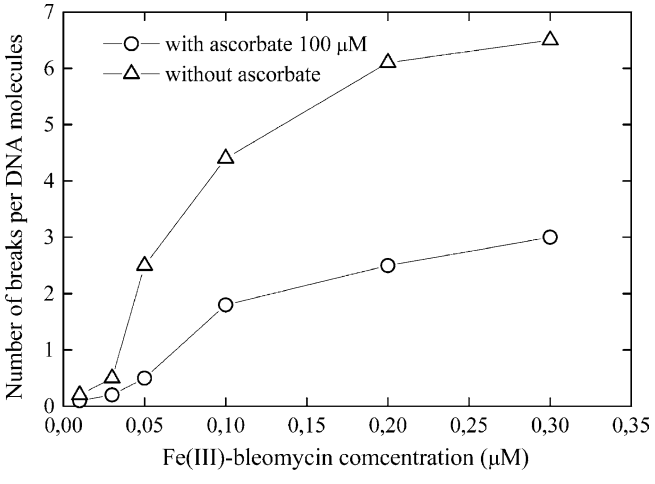

Fig. 6 Plot of the number of DNA breaks per molecule versus the Fe(III)-bleomycin concentration for molecules adsorbed onto mica prior to interacting with bleomycin, with and without 100 μM ascorbate. The number of DNA breaks does not depend linearly on the bleomycin bulk concentration (which is observed in bulk), which indicates that the surface influences the cleavage of DNA. In the absence of ascorbate, the adsorbed molecules are damaged more than in the bulk. The amplification of the DNA cleavage by ascorbate is also less efficient than in the bulk

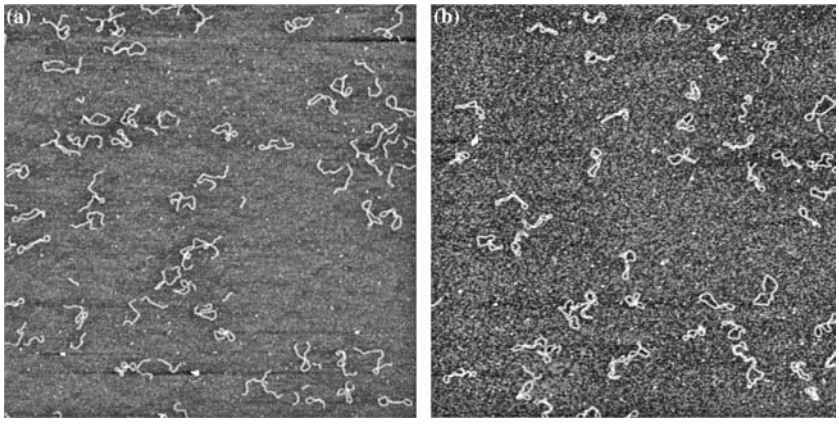

Fig. 7 Images of DNA molecules after adsorption and interaction with Fe(III)-bleomycin. The DNA molecules were previously adsorbed by adding $NiCl_2$ to the deposition buffer. 200 nM Fe(III)-bleomycin was then allowed to interact with DNA for 3 min. **a** 1 µl of 1 mM $NiCl_2$ was added to 4 µl of DNA deposition buffer. **b** 1 µl of 30 mM $NiCl_2$ was added to 4 µl of DNA deposition buffer. It is apparent that DNA cleavage is strongly inhibited by the addition of $NiCl_2$, which neutralizes the DNA surface (this inhibition is also important when the mica is pre-treated with $NiCl_2$ and dried before DNA deposition). Also, the final concentration of $NiCl_2$ during incubation with bleomycin is relatively low (diluted 25-fold) since 20 µl of the buffer containing bleomycin is added to the DNA deposition buffer on the mica surface. Ni^{2+} ions cannot effectively compete with bleomycin for DNA binding (control experiment not shown). Scan area: 5×5 μm^2

DNA by a factor of eight, as it has been observed in bulk (Buettner and Moseley 1992; Sugiyama et al. 1986). On the surface of the mica, the number of DNA breaks is weakly enhanced by a factor of about three. The negative charge of the ascorbate molecule may be repelled by the negatively-charged surface so that the enhancement is weaker than expected. Nevertheless, the main reason could be that the amount of DNA substrate is small compared to the high surface concentration of adsorbed bleomycin. This suggests that the catalytic function of Fe(III)-bleomycin may be less enhanced by ascorbate (Ajmera et al. 1986).

The cleavage of DNA by bleomycin is saturated at lower bleomycin concentrations on the surface than in the bulk (with or without ascorbate). The Mg^{2+} concentration in the condensed layer is very high (see Fig. 3), which may inhibit the bleomycin binding. In addition, high ionic strength is known to sharply increase the size of the bleomycin-binding site on DNA (Chien et al. 1977; Huang et al. 1980) Therefore the counterion layer could prevent the binding of bleomycin to DNA adsorbed on a mica surface.

Figure 7 shows two AFM images of DNA molecules for two $NiCl_2$ concentrations (200 µM and 6 mM). Figure 8 shows the number of DNA breaks per molecule versus the concentration of the 1 µl of $NiCl_2$ solution added to the DNA deposition buffer. Ni^{2+} ions are exchanged with the potassium ions on the surface, which leads to charge neutralization and a charge reversal at high concentrations of Ni^{2+} ions. We can see that DNA is weakly damaged for $NiCl_2$ concentrations larger than 10 mM. This indicates that the surface charge inversion strongly inhibits DNA cleavage by bleomycin on the surface (this inhibition is also obtained for mica surfaces pre-treated with $NiCl_2$ solution, rinsed and dried before DNA deposition). When 1 µl of 1 mM $NiCl_2$ solution is added to the deposition buffer, the 2×10^{18} m^{-2} mica surface sites are partly neutralized by the Ni^{2+} ions since a surface area of mica of about 2 cm^2 is used for deposition (so there are 4×10^{14} negatively-charged mica sites and 6×10^{14} Ni^{2+} ions). The Ni^{2+} concentration at which DNA cleavage is inhibited is roughly the concentration at which the mica surface starts to be neutralized (see Fig. 6). At lower concentrations of $NiCl_2$ (< 1 mM), the mica surface charge does not change significantly, and the presence of Ni^{2+} does not influence the bleomycin activity either. In this respect, the bleomycin can be considered to be a probe of the charge density on the surface onto which the DNA is adsorbed.

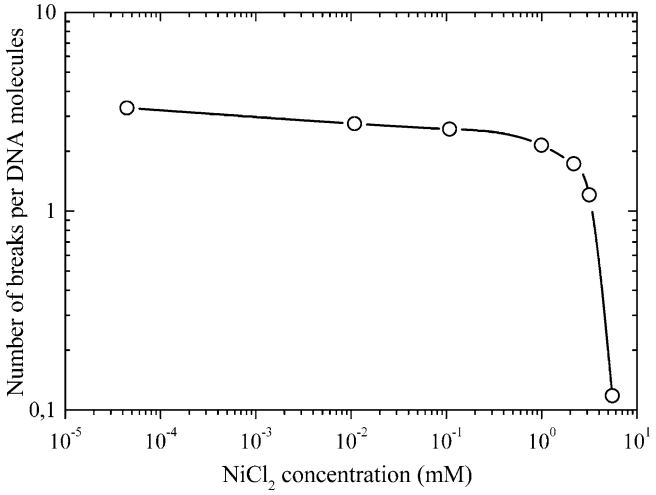

Fig. 8 Plot of the number of DNA breaks per adsorbed molecule for interactions with 200 nM Fe(III)-bleomycin versus the $NiCl_2$ concentration added to the deposition buffer (see caption of Fig. 7). It appears that the Ni^{2+} ions strongly adsorbed onto the mica surface inhibit the DNA cleavage, possibly due to the change in the surface charge induced by the adsorption of Ni^{2+}

The measurement of the cleavage efficiency of Fe(III)-bleomycin on the mica surface reveals that the DNA surface adsorption does not inhibit the DNA cleavage by bleomycin when the surface is negatively-charged (untreated). The electrostatic adsorption of DNA on mica mediated by Mg^{2+} divalent counterions is weak, which allows the bleomycin cleavage mechanism to take place. However the experimental results show that the interaction between bleomycin and adsorbed DNA is rather different to that in bulk. In particular, the reaction takes place in the condensed counterion layer on the negatively-charged mica surface. The bleomycin molecules can therefore be considered as counterions that participate in the surface neutralization. This suggests that the surface concentration of bleomycin should be larger on the mica surface than in bulk. The number of DNA breaks per molecule as measured by AFM is larger on the surface than in the bulk, but it is not as high as we might expect if we consider the high surface concentration of Fe(III)-bleomycin. This indicates that the cleavage of DNA by bleomycin is less effective for adsorbed DNA molecules, which is partly due to the high concentration of divalent counterions, but may also be because the DNA molecules adsorbed on the mica are not as accessible.

The surface charge is crucial to bleomycin cleavage, which is strongly inhibited when the surface charge is reversed by adding $NiCl_2$ to the deposition buffer. The results obtained from these experiments allow us to put forward some hypotheses to explain the effects of the surface charge. There is an electrostatic repulsion or attraction between bleomycin and the positively- or negatively-charged mica surface, respectively. However, we cannot exclude the role played by the orientations of the bleomycin molecules adsorbed on the surface, which can influence the DNA breakage. It is possible that the molecular orientation depends on the surface charge. In addition, the binding of DNA to the positively-charged surface is enhanced. Molecular rearrangements are hindered and the molecules adopt a more compact conformation (Pastré et al. 2003), which can make the cleavage of the DNA molecule more difficult. The experiments conducted in this study clearly demonstrate that the nature of the surface charge can perturb the cleavage of DNA by bleomycin. Similar AFM studies need to be carried out while studying DNA/ligand interactions. We have also provided evidence for the inhibition of anti-tumoral activity when DNA is bound to a positively-charged surface, which may be of particular interest when studying the action of bleomycin in chromatin (Cuiffo et al. 1985; Lönn et al. 1990; Smith et al. 1994).

References

Abraham AT, Zhou X, Hecht SM (1999) DNA Cleavage by Fe(II)·Bleomycin conjugated to a solid support. J Am Chem Soc 121:1982–1983

Ajmera S, Wu JC, Worth L, Jr, Rabow LE, Stubbe J, Kozarich JW (1986) DNA degradation by bleomycin: evidence for 2′R-proton abstraction and for C-O bond cleavage accompanying base propenal formation. Biochemistry 25:6586–6592

Allison DP, Kerper PS, Doktycz MJ, Spain JA, Modrich P, Larimer FW, Thundat T, Warmack RJ (1996) Direct atomic force microscope imaging of EcoRI endonuclease site specifically bound to plasmid DNA molecules. Proc Natl Acad Sci USA 93:8826–8829

Berge T, Jenkins NS, Hopkirk RB, Waring MJ, Edwardson JM, Henderson RM (2002) Structural perturbations in DNA caused by bis-intercalation of ditercalinium visualised by atomic force microscopy. Nucleic Acids Res 30:2980–2986

Biggins JB, Prudent JR, Marshall DJ, Ruppen M, Thorson JS (2000) A continuous assay for DNA cleavage: the application of "break lights" to enediynes, iron-dependent agents, and nucleases. Proc Natl Acad Sci USA 97:13537–13542

Buettner GR, Moseley PL (1992) Ascorbate both activates and inactivates bleomycin by free radical generation. Biochemistry 31:9784–9788

Burger RM (1998) Cleavage of nucleic acids by bleomycin. Chem Rev 98:1153–1170

Cary RB, Peterson SR, Wang J, Bear DG, Bradbury EM, Chen DJ (1997) DNA looping by Ku and the DNA-dependent protein kinase. Proc Natl Acad Sci USA 94:4267–4272

Chien M, Grollman AP, Horwitz SB (1977) Bleomycin-DNA interactions: fluorescence and proton magnetic resonance studies. Biochemistry 16:2641–2647

Claussen CA, Long EC (1999) Nucleic acid recognition by metal complexes of bleomycin. Chem Rev 99:2797–2816

Cuiffo BP, Fox HB, Babior BM (1985) Chromatin structure during bleomycin-induced DNA damage and repair. Free Radical Biol Med 1:139–144

D'Andrea AD, Haseltine WA (1978) Sequence specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin. Proc Natl Acad Sci USA 75:3608–3612

Gier S, Johns WD (2000) Heavy metal-adsorption on micas and clay minerals studied by X-ray photoelectron spectroscopy. Appl Clay Sci 16:289–299

Guthold M, Bezanilla M, Erie DA, Jenkins B, Hansma HG, Bustamante C (1994) Following the assembly of RNA polymerase-DNA complexes in aqueous solutions with the scanning force microscope. Proc Natl Acad Sci USA 91:12927–12931

Huang CH, Galvan L, Crooke ST (1980) Interactions of bleomycin analogues with deoxyribonucleic acid and metal ions studied by fluorescence quenching. Biochemistry 19:1761–1767

Koppelman MH, Dillard JD (1977) A study of the adsorption of Ni(II) and Cu(II) by clay minerals. Clay Clay Miner 73:457–462

Li W, Xia C, Antholine WE, Petering DH (2001) Interactions of iron bleomycin, phosphate or cyanide, and DNA: sequence-dependent conformations and reactions. J Biol Inorg Chem 6:618–627

Li W, Antholine WE, Petering DH (2002) Kinetics of reaction of DNA-bound Fe(III)bleomycin with ascorbate: interplay of specific and non-specific binding. J Inorg Biochem 90:8–17

Lloyd RS, Haidle CW, Robberson DL (1978) Bleomycin-specific fragmentation of double-stranded DNA. Biochemistry 17:1890–1896

Lloyd RS, Haidle CW, Robberson DL (1979) Noncovalent intermolecular crosslinks are produced by bleomycin reaction with duplex DNA. Proc Natl Acad Sci USA 76:2674–2678

Lönn U, Lönn S, Nylen U, Windblad G (1990) Bleomycin-induced DNA lesions are dependent on nucleosome repeat length. Biochem Pharmacol 39:101–107

Lyubchenko YL, Jacobs BL, Lindsay SM, Stasiak A (1995) Atomic force microscopy of nucleoprotein complexes. Scanning Microscopy 9:705–724

Nagami F, Zuccheri G, Samori B, Kuroda R (2002) Time-lapse imaging of conformational changes in supercoiled DNA by scanning force microscopy. Anal Biochem 300:170–176

van Noort SJ, van der Werf KO, Eker AP, Wyman C, de Grooth BG, van Hulst NF, Greve J (1998) Direct visualization of dynamic

protein-DNA interactions with a dedicated atomic force microscope. Biophys J 74:2840–2849

Pashley RM, Israelachvili JN (1984) DLVO and hybridation forces between mica surfaces in Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} chloride solutions. J Colloid Interf Sci 97:446–455

Pastré D, Piétrement O, Fusil S, Landousy F, Jeusset J, David MO, Hamon L, Le Cam E, Zozime A (2003) Adsorption of DNA to mica mediated by divalent counterions: a theoretical and experimental study. Biophys J 85:2507–2518

Petering DH, Byrnes RW, Antholine WE (1990) The role of redox-active metals in the mechanism of action of bleomycin. Chem Biol Interact 73:133–182

Piétrement O, Pastré D, Fusil S, Jeusset J, David M-O, Landousy F, Hamon L, Zozime A, Le Cam E (2003) Reversible binding of DNA on $NiCl_2$ treated mica by varying the ionic strength. Langmuir 19:2536–2539

Pope LH, Davies MC, Laughton CA, Roberts CJ, Tendler SJ, Williams PM (2000) Atomic force microscopy studies of intercalation-induced changes in plasmid DNA tertiary structure. J Microsc 199:68–78

Povirk LF, Goldberg IH (1983) Stoichiometric uptake of molecular oxygen and consumption of sulfhydryl groups by neocarzinostatin chromophore bound to DNA. J Biol Chem 258:11763–11767

Povirk LF, Houlgrave CW (1988) Effect of apurinic/apyrimidinic endonucleases and polyamines on DNA treated with bleomycin and neocarzinostatin: specific formation and cleavage of closely opposed lesions in complementary strands. Biochemistry 27:3850–3857

Revet B, Fourcade A (1998) Short unligated sticky ends enable the observation of circularised DNA by atomic force and electron microscopies. Nucleic Acids Res 26:2092–2097

Rouzina I, Bloomfield VA (1996a) Competitive electrostatic binding of charged ligands to polyelectrolytes: planar and cylindrical geometries. J Phys Chem 100:4292–4304

Rouzina I, Bloomfield VA (1996b) Influence of ligand spatial organization on competitive electrostatic binding to DNA. J Phys Chem 100:4305–4313

Sakai TT, Riordan JM, Glickson JD (1983) Bleomycin interactions with DNA. Studies on the role of the C-terminal cationic group of bleomycin A2 in association with and degradation of DNA. Biochim Biophys Acta 758:176–180

Smith BL, Bauer GB, Povirk LF (1994) DNA damage induced by bleomycin, neocarzinostatin, and melphalan in a precisely positioned nucleosome. Asymmetry in protection at the periphery of nucleosome-bound DNA. J Biol Chem 269:30587–30594

Steighner RJ, Povirk LF (1990) Bleomycin-induced DNA lesions at mutational hot spots: implications for the mechanism of double-strand cleavage. Proc Natl Acad Sci USA 87:8350–8354

Sugiyama H, Morii T, Saito I, Matsuura T, Hecht SM (1986) Structure and chemistry of alkali-labile product formed during Fe(II)-bleomycin-mediated DNA strand scission. Nucleic Acids Symp Ser 17:165–166

Takeshita M, Grollman AP, Horwitz SB (1976) Effect of ATP and other nucleotides on the bleomycin-induced degradation of vaccinia virus DNA. Virology 69:453–463

Takeshita M, Grollman AP, Ohtsubo E, Ohtsubo H (1978) Interaction of bleomycin with DNA. Proc Natl Acad Sci USA 75:5983–5987

Utsuno K, Tsuboi M, Katsumata S, Iwamoto T (2001) Viewing of complex molecules of ethidium bromide and plasmid DNA in solution by atomic force microscopy. Chem Pharm Bull 49:413–417

Utsuno K, Tsuboi M, Katsumata S, Iwamoto T (2002) Visualization of complexes of Hoechst 33258 and DNA duplexes in solution by atomic force microscopy. Chem Pharm Bull 50:216–219