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Interaction of Zn(II)Bleomycin-A₂ and Zn(II)Peplomycin with a DNA Hairpin containing the 5'-GT-3' Binding Site in Comparison with the 5'-GC-3' Binding Site Studied by NMR Spectroscopy

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Abstract

Bleomycins are a group of glycopeptide antibiotics synthesized by *Streptomyces verticillus* that are widely used for the treatment of various neoplastic diseases. These antibiotics have the ability to chelate a metal center, mainly Fe(II), and cause site-specific DNA cleavage. Bleomycins are differentiated by their C-terminal regions. Although this antibiotic family is a successful course of treatment for some types of cancers, it is known to cause pulmonary fibrosis. Previous studies have identified that bleomycin-related pulmonary toxicity is linked to the C-terminal region of these drugs. This region has been shown to closely interact with DNA. We examined the binding of Zn(II)peplomycin and Zn(II)bleomycin-A₂ to a DNA hairpin of sequence 5'-CCAGTATTTTACTGG-3', containing the binding site 5'-GT-3', and compare the results with those obtained from our studies of the same MBLMs bound to a DNA hairpin containing the binding site 5'-GC-3'. We provide evidence that the DNA base sequence has a strong impact in the final structure of the drug-target complex.

Keywords

DNA; NMR; pulmonary fibrosis; anticancer drug; structure-function

Introduction

The antitumor antibiotics Bleomycins (BLMs), first discovered in 1966 by H. Umezawa [1], constitute a family of glycopeptides isolated from cultures of *Streptomyces verticillus* [1]. BLMs are used for the treatment of several types of tumors [2–5], based on their ability to bind a Fe(II) ion and selectively cleave DNA. The BLM structure is separated into three main domains with different functions (Fig. 1). Despite the efficiency of BLMs as chemotherapeutic agents, BLM-related pulmonary toxicity [6] has hindered the use of the drug on cancer patients. Rodent models developed for the study of pulmonary fibrosis upon BLM administration [7–15] have indicated that the severity of BLM-induced lung injury in mice can be attributed to the C-termini (tails), which differentiate BLMs. Over 300

bleomycin analogs varying in the terminal C-substituent have been developed in an attempt to alleviate the pulmonary toxicity effects of this drug [7,10,12–14,16].

In the last decade, the DNA-binding domain in BLM (Fig. 1) has been the subject of some controversy regarding its mode of interaction with DNA. Various studies have led to the proposal of three different modes of binding of metallo-BLMs (MBLMs) to DNA, including minor groove binding [17–19], and partial [20–22] and total [23] intercalation of the bithiazole (Bit) moiety to the DNA double helix. However differences in the DNA base sequences, as well as the metal centers and BLMs used in these studies, have made it difficult to consolidate the research results leading to a consensus. Additionally, the main focus of these studies has been the interaction of the Bit moiety with the DNA fragment, but the effect that the BLM tail has on the DNA structure upon MBLM complexation has been neglected. If the toxicity of BLM is related to the BLM tails, it seems reasonable to hypothesize that the binding interaction of MBLMs with DNA may be different for each C-terminus. Previous studies have focused on the two known DNA cleavage sites that BLMs are known to interact with, mainly 5'-GC-3' and 5'-GT-3'[24], however a preference of cleavage site is not well understood [19,25]. The interactions of different MBLMs with DNA including Fe(II)- [26–28], Zn(II)- [17,18], Co(III)- [6,20–23,29–34], and Cu(I)-bound BLMs [35], have been examined. However the possibility of different coordination geometries of these metal centers to BLM and their repercussions on the final structure of the MBLM-DNA triad have received little attention. It is reasonable to assume that factors such as metal center, DNA base sequence, and BLM C-termini could have an impact on the mode of binding of MBLMs to DNA. Therefore, studies on this area need to be designed to control each factor separately in order to establish the relative significance of each portion of the drug, and to hopefully improve the therapeutic efficiency of BLMs.

We have previously studied the conformations of Zn(II)BLM-DNA triads [36], focusing on four different BLM C-termini, and examined the impact of the binding of these MBLMs on the structure of a DNA hairpin containing the 5'-GC-3' binding site (OL₁). The MBLM proposed to cleave DNA *in vivo* is Fe(II)BLM [37], however, Fe(II)BLM complexes are paramagnetic [38–42], making the study of their solution conformation through NMR very difficult. Additionally, Fe(II)BLM is highly sensitive to oxidation, and could cleave DNA in the presence of traces of O₂. For these reasons, the selected metal center for these studies was Zn²⁺, due to its diamagnetic character and inability to become activated and prompt DNA cleavage by BLM, thereby guaranteeing the integrity of the oligonucleotide, and facilitating NMR data analysis. Additionally, the ligands to the metal center reported for Zn(II)BLM [31] are the same found by us in our extensive studies of Fe(II)BLM complexes [38–42]. The DNA base sequence was selected to be very similar to that used on the study of HOO-CoBLM-A₂ green bound to a DNA fragment [21], with the aim of comparing our results to those obtained by Wu *et al.* Due to the length of the oligonucleotide, and its self-complementarity, we decided to use DNA hairpins in order to guarantee that the BLM-binding site was located in a double-stranded region of the DNA segment, while keeping the oligonucleotide at minimum complexity for the sake of NMR data analysis. The selected hairpin still contains the important inter-strand interactions found in double-stranded DNA, and therefore is a valid test model. The results of the aforementioned studies suggested that Zn(II)-peplomycin (Zn(II)PEP) produced the lowest degree of disturbance of the initial

structure (free DNA) of the hairpin, whereas Zn(II)BLM-A₅ had the highest degree of disturbance. These two BLMs are at the opposite ends of the spectrum of pulmonary toxicities [6,8–15,43,44] displayed by this family of antibiotics. The differences in the binding interactions of the BLM tails could potentially be related to the toxicity level of each BLM, although more research would be required to reach solid conclusions regarding this point. The goal of the present study is to examine the binding of Zn(II)PEP and Zn(II)BLM-A₂ to a DNA hairpin of sequence 5'-CCAGTATTTTACTGG-3' (OL₂), containing the binding site 5'-GT-3', and compare the results with those obtained from our studies of the same MBLMs bound to a DNA hairpin containing the binding site 5'-GC-3' [36]. The proposed comparison could contribute important elements regarding the influence that the binding site in DNA has on the conformation of the triads. Systematic research on the mode of binding of the metal complexes of BLMs to DNA is required to understand the specifics about BLM-derived pulmonary damage, and hopefully engineer BLMs with higher therapeutic index.

Materials and methods

Materials

BLM-A₂ was purchased from TOKU-E (Bellingham, WA). PEP was a generous gift of Nippon Kayaku Co., Ltd. (Tokyo, Japan). Zinc sulfate 7-hydrate was purchased from VWR (Radnor, PA). Deuterated water (99.9%, d), sodium hydroxide, and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO). The DNA hairpin was purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

MBLMs binding constant determination

Both the extinction coefficients for Zn(II)PEP and Zn(II)BLM-A₂ and the binding constants for Zn(II)PEP and Zn(II)BLM-A₂ bound to OL₁ and OL₂ were determined using UV-Visible spectrometry (UV-Vis 420; Aviv Biomedical, Lakewood, NJ). The extinction coefficients at 292 nm were determined experimentally for Zn(II)PEP ($\epsilon = 17,048 \text{ M}^{-1}\text{cm}^{-1}$) and Zn(II)BLM-A₂ ($\epsilon = 31,499 \text{ M}^{-1}\text{cm}^{-1}$) using standard procedures. The extinction coefficients for the OLs were determined based on their base sequences using the Integrated DNA Technologies prediction tools (OL₁: $\epsilon = 23,974.3 \text{ M}^{-1}\text{cm}^{-1}$ at 292 nm, $\epsilon = 122,000 \text{ M}^{-1}\text{cm}^{-1}$ at 260 nm; OL₂: $\epsilon = 23,788.9 \text{ M}^{-1}\text{cm}^{-1}$ at 292 nm, $\epsilon = 149,700 \text{ M}^{-1}\text{cm}^{-1}$ at 260 nm) [45]. The equilibrium/competitive dialysis procedure used is based on published protocols [46–49]. Due to the low absorbance of the BLMs, higher concentrations were used, 400 mL of 10 μM Zn(II)BLM (1:1 molar ratio of Zn₂SO₄ and BLM) were prepared in H₂O with a pH value of 6.5. OL samples were prepared for a range of concentrations (50, 75, 100, 125, and 150 μM) with 20 mM NaCl in 100 μL of H₂O with a pH value of 6.5. Slide-A-Lyzer™ MINI dialysis devices (ThermoFisher Scientific) with a molecular cut off of 3.5 kDa and a sample volume of 10–100 μL were used to perform the experiment. Each OL sample was put in an individual dialysis device and placed in a flotation device within a 1 L beaker containing the 10 μM Zn(II)BLM and then covered with parafilm and placed on a stir plate. The samples were allowed to equilibrate over a 24 hour period at room temperature (22–24 °C) with continuous mixing. After dialysis, the samples were removed from the dialysis devices, diluted to 300 μL and sodium dodecyl sulfate (SDS) was added to a final

concentration of 1% (w/v). UV-Visible spectra were acquired for all OL samples and the free Zn(II)BLM (ligand in the beaker). The calculations took into account the dilution of the OL samples when determining the actual concentrations. Equation (1) was used to determine the binding constants [47]:

$$K_A = \frac{[bound\ Zn(II)BLM]}{[free\ Zn(II)BLM][OL]} \quad (1)$$

based on the chemical equation (2)

The concentration of Zn(II)BLM complexed with OL was determined using equation (3) [47]:

$$[bound\ Zn(II)BLM] = [Zn(II)BLM\ in\ dialysis\ device] - [Zn(II)BLM\ in\ beaker] \quad (3)$$

NMR Sample Preparation

BLM-A₂ and PEP samples, 1.95 μmol, were dissolved in 650 μL of D₂O. A 0.12 M solution of ZnSO₄·7H₂O was mixed with the BLM solution to achieve a 1:1 molar ratio of Zn(II):BLM. The pH was adjusted to approximately 6.5 with a 0.1 M NaOD solution (pH meter reading uncorrected for the deuterium isotope effect). DNA samples, 0.335 μmol, were dissolved in 603 μL D₂O and 67 μL of a 200 mM NaCl solution was added. The pH adjusted to approximately 6.5 (pH meter reading uncorrected for the deuterium isotope effect). The Zn(II)BLM solutions were titrated with the DNA samples until a 1:1 molar ratio for the Zn(II)BLM:DNA complex was achieved. One-dimensional (1D) ¹H-NMR spectra were used to monitor the changes in the complex formation. There were no additional changes in the 1D spectra once a 1:1 molar ratio was achieved. Zn(II)BLM and DNA samples in 90% H₂O/10% D₂O (referred to as spectra in H₂O) were prepared by analogous procedures.

NMR Spectra Collection

NMR spectra were acquired on a 600 MHz Bruker AVANCE III 600 spectrometer (Bruker BioSpin Corp, Billerica, MA) with a 5.0 mm multi-nuclear broad-band observe probe. Spectra were acquired at both 278 K and 298 K for all samples and were referenced to HDO and H₂O as internal standards. Two-dimensional (2D) experiments including total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) were acquired with solvent suppression achieved by excitation sculpting with gradients. The mixing times for the TOCSY and NOESY experiments were 200 ms and 40 ms, respectively. The spectral width was set to 10 ppm for D₂O samples and 20 ppm for H₂O samples (in both dimensions), and 512 t₁ points were acquired with 2048 complex points for each free induction decay (FID). The number of scans for a t₁ point for the TOCSY and

NOESY experiments were 32 and 48, respectively. All spectra were Fourier transformed using Lorentzian-to-Gaussian weighting and phase-shifted sine-bell window functions. NMR spectra were processed and analyzed using Topspin3.0 and NMR ViewJ software.

Results and Discussion

The main goal of the research presented herein is to assess the effect of the DNA binding site on the structure of triads formed between OL₂ (5'-GT-3' binding site) and OL₁ (5'-GC-3' binding site [36]) and various Zn(II)BLMs. To ensure the presence of double stranded DNA, both studies involved DNA hairpin loops with a similar design. The two DNA sequences differ on their binding sites and also in the base pair content. OL₂ can be considered AT rich (3 AT base pairs) compared to OL₁ (1 AT base pair). Fig. 2 shows the changes that occur in the imino region of OL₁ [36] and OL₂ upon binding of Zn(II)BLM-A₂ and Zn(II)PEP. Table 1 compiles the differences in chemical shift between the exchangeable protons in free and MBLM-bound OL₂. Broadening and shifting of the OL₂ signals in this region gives a strong indication that MBLM binding is occurring. For OL₂, complexation with both MBLMs causes similar downfield shifting of the imino signals, with Zn(II)PEP producing a greater degree of broadening. Comparison of the left and right panels in Fig. 2 indicates that OL₂ exhibits broadening of the imino signals to a much greater extent for both MBLMs than previously observed in the partner triads with OL₁ [36]. On the other hand, the imino signals exhibit similar downfield shifting in the presence of the MBLMs for both hairpins [36].

In order to further our understanding of the OLs exchange regimes, titrations of both oligonucleotides with MBLMs were performed to estimate the exchange rate of OL₁ [36] and OL₂ (Supplementary Figure S1) between their free and MBLM-bound forms. The NMR data collected in both studies indicate an intermediate exchange rate at 25 °C. This fact is evinced by the diminishing intensity of some of the NMR signals generated by the free OLs, as aliquots of the Zn(II)BLMs are added during the titration experiments. Additionally, new signals are observed as the MBLM:DNA molar ratio approaches 1:1. The dissociation constants of Zn(II)PEP and Zn(II)BLM-A₂ from both OLs were measured over a series of DNA concentrations. These constants were found to be for Zn(II)PEP: $K_d = 1.5 \times 10^{-5}$ M when bound to OL₁ and $K_d = 6.7 \times 10^{-6}$ M when bound to OL₂; and for Zn(II)BLM-A₂: $K_d = 4.3 \times 10^{-6}$ M when bound to OL₁, and $K_d = 3.1 \times 10^{-6}$ M when bound to OL₂. These results suggest tighter binding of Zn(II)PEP and Zn(II)BLM-A₂ to OL₂. The favoring of AT-rich regions in DNA by Zn(II)BLMs has been pointed out by various researchers in the field [17,18]. Dissociation constants have been reported for other MBLM-DNA complexes. K_{ds} for Co(III)BLM-A₂ bound to both 5'-GC-3' and 5'-GT-3' binding sites have been 1.7×10^{-7} M and 1.5×10^{-7} M, respectively [31,33]. Whereas, metal free BLM-A₂ has been reported to have K_{ds} of 8.3×10^{-6} M [50] and 2×10^{-6} M [51]. The dissociation constants reported here are within similar range as those previously reported, with differences possibly caused by the different metal centers bound to BLM-A₂. This information confirms what we have seen through our NMR studies in comparison of the two preferential binding sites. Zn(II)BLMs bound to both OLs are at intermediate exchange rates, with the drugs spending more time attached to OL₂ than to OL₁. With a smaller dissociation constant, Zn(II)BLMs are bound tighter to OL₂ than OL₁, which has an impact on the rate of exchange and causes the imino

signals shown in Fig. 2 to be broader for OL₂ than OL₁. It is worth noticing that the binding constants of the MBLMs considered herein are different, suggesting that the BLM C-termini have an effect on the strength of the binding to the oligonucleotides.

Regarding the shifts displayed by the imino signals in OL₂ upon MBLM binding, Table 1 shows that mostly downfield shifts are detected for these signals. As previously pointed out by Feigon *et al.* [52], DNA-intercalators generally produce upfield shifting of the DNA-imino ¹H signals. The shifts observed for the imino signals generated by OL₂ are more in agreement with binding of the drugs in a groove of the oligonucleotide [52]. The same trend was observed for the imino signals in OL₁ bound to the same MBLMs [36]. Fig. 3 shows schematic representations of the bases whose exchangeable-proton signals experience significant shifting (δ greater than or equal to |0.04| ppm) upon MBLM complexation for OL₁ [36] and OL₂. As it can be seen in this figure, more native inter-base signals (imino/amino NMR signals) are disturbed in OL₂ when bound to Zn(II)PEP and Zn(II)BLM-A₂ than in the OL₁ partner triads. Additionally, in the OL₂ triads base-pair contacts distant from the 5'-GT-3' binding site are also disturbed, indicating their possible rearrangement to accommodate the drugs. The same rearrangement is very mild in OL₁, involving only the C13 base. This result suggests that besides the BLM C-termini, the oligonucleotide base sequence has an impact in the interaction between the drug and its target.

Upon complexation of the MBLMs to OL₂, the Bit and β -hydroxyhistidine (Hist) signals in the MBLMs also experience broadening and shifting, which is another indication that binding to the hairpin is taking place. Fig. 4 shows the aromatic region of the 1D ¹H-NMR spectra for both free and OL₂-bound Zn(II)BLMs. The difference chemical shifts (δ) and peak width (ω) for the Bit and Hist signals for both BLMs under study in the presence of OL₂ are collected in Supplementary Table S1. Examination of the Bit and Hist signals in these spectra and in Supplementary Table S1 indicates that the Bit signals have a greater upfield shift and more extensive broadening in OL₂-bound Zn(II)PEP than in the OL₂-Zn(II)BLM-A₂ triad. One of the Hist signals experience similar upfield shifting in both triads. The extent of broadening of the other Hist signal is greater for the OL₂-Zn(II)BLM-A₂ triad than for the partner Zn(II)PEP triad. Although the Bit and Hist signals in the presence of OL₁ [36] and OL₂ both display broadening and upfield shifting, there are differences in the trends of these parameters when in the presence of these two hairpins. Besides indicating the binding of MBLMs to DNA, the shifting and broadening of the Hist and Bit signals in bound MBLM have been used as an evidence of interactions between MBLM and its target DNA [17]. As determined in our investigation of OL₁ bound to four different MBLMs, these signals behave differently depending on the MBLM bound [36]. The results presented herein (Fig. 4 and Supplementary Table S1) indicate that the Bit signals in Zn(II)PEP bound to OL₂ display greater upfield shifts and broadening than those in the OL₂-Zn(II)BLM-A₂ triad. The opposite result was observed for Zn(II)PEP and Zn(II)BLM-A₂ bound to OL₁ [36]. The detected Hist signals in the Zn(II)BLM-A₂ and Zn(II)PEP shifted similarly upfield upon binding to OL₂, with more broadening displayed by these signals in the OL₂-Zn(II)BLM-A₂ triad. This trend was again the inverse of that observed in the partner OL₁ triads. These results reinforce the notion that the DNA base sequence has a strong influence on the mode of interaction between MBLM and DNA.

In order to further examine the effect that the Zn(II)BLMs have on the inter-strand structure of OL₂, NOESY spectra were acquired for samples in H₂O at 5 °C for free and Zn(II)BLM-bound OL₂. NOEs for the exchangeable protons observed for free OL₂ are labeled and presented in Fig. 5, with the corresponding signal assignments listed in Supplementary Table S2. Fig. 5A shows the overlay of the spectra acquired for free OL₂ in H₂O and D₂O at 5 °C. Figs. 5B and 5C show the NOEs exhibited by the imino protons. The DNA base sequence of OL₂ includes three AT base-pairs, therefore exhibiting cross peaks between the T and G imino protons (Fig. 5C). Additionally, the T imino protons also have cross peaks with their corresponding methyl protons (Fig. 5D). As previously determined for various Zn(II)BLM-OL₁ triads [36], upon complexation with Zn(II)BLM-A₂ and Zn(II)PEP, we observe some of the signals from the amino and imino protons in OL₂ shift from their original positions for free OL₂. Overlays of the 5 °C NOESY spectra collected for samples in H₂O for free and MBLM-bound OL₂ are shown in Figs. 6 (amino region) and 7 (imino region). The chemical shift differences (δ s) for the imino and amino proton signals compared to those of free OL₂ are displayed in Table 1. In the presence of both Zn(II)BLMs, there is significant shifting (δ greater than or equal to |0.04| ppm) of one of the protons of the C2NH₂, the A3NH₂ and A12NH₂ groups, and the T11NH proton. The magnitude of these shifts being greater for the Zn(II)BLM-A₂-OL₂ triad. The bases located close to the 3' and 5' ends of the hairpin are affected differently depending on the MBLM bound. The observed shifts of the amino signals in the OL₂ triads are comparable to the ones previously observed for the MBLM-OL₁ partner triads [36]. On the other hand, in the MBLM-OL₂ triads studied herein more DNA bases exhibit these shifts (Fig. 3), 7 bases affected in OL₂ triads vs. 4 bases affected in OL₁ triads [36].

A significant loss in the number of free-OL₂ NOEs is observed in the imino and amino regions of the NOESY spectra upon triad formation, indicating that the distances between the corresponding protons become greater than 5 Å upon MBLM complexation. Interestingly, there are also new NOEs between OL₂ protons found in these regions of the spectra upon triad formation. New NOEs were not identified in our studies of Zn(II)BLMs-OL₁ partner triads for the exchangeable protons, and can indicate a significant change in the inter-strand structure of OL₂. Supplementary Table S2 includes the free-OL₂ NOEs that are lost upon MBLM complexation, as well as the new NOEs found. Schematic diagrams showing the NOEs detected for free OL₂ (Supplementary Figure S2), and the NOEs from free OL₂ that are conserved upon MBLM complexation (conserved NOEs) plus the NOEs that arise between OL₂ protons upon MBLM complexation (Fig. 8) are provided for easy visualization. Zn(II)BLMs have been depicted binding OL₂ as suggested by Wu *et al.* [21]. Some of the new NOEs shown in Fig. 8 are connections between protons in bases C2 and C13 of the hairpin, and suggest that a significant alteration of the native structure of OL₂ is taking place, bringing these two bases close enough to result in NOEs. These new inter-strand NOEs could be interpreted to indicate that both Zn(II)BLMs appear to cause a kink in the DNA that results in the C2 and C13 bases being closer than in the native OL₂. Distortions (bends/kinks) of the structures of oligonucleotides bound to Zn(II)BLMs have been previously reported [17] Interestingly, the PEP triad exhibits 6 new NOE connections while the A₂ triad displays 9 of them. This fact indicates that, as previously observed for Zn(II)BLM triads with OL₁ [36], each BLM affects the DNA segment differently.

Regarding the free-OL₂ NOEs that are conserved after Zn(II)BLM complexation, Fig. 8 shows that most of the connections in the region above the binding site are lost, indicating that the BLM is disrupting the interior of the hairpin above the binding site to a greater extent than below the binding site. Overall out of 62 inter-strand NOEs detected for free OL₂, the PEP triad conserves 31% and the A₂ triad conserves 35% of them for the exchangeable protons. This is a significant difference compared to the Zn(II)PEP-OL₁ complex, which conserved 64% of the free-OL₁ NOEs in this region, whereas the Zn(II)BLM-A₂-OL₁ complex conserved 43% of these NOEs. The conserved NOEs for OL₁ are equally distributed along the interior of the hairpin [36], whereas these NOEs for OL₂ remain mainly below the binding site. These results are an indication that the inter-strand native structure of OL₂ is in general disrupted to a greater extent than that of OL₁ as a consequence of MBLM binding. Additionally, the 5'-GT-3' binding site seems to undergo significant restructuring in the presence of the drugs, as indicated by the loss of the NOEs between the base pairs A6NH₂-T11NH and A12NH₂-T5NH protons.

The external core of OL₂, represented by the non-exchangeable base protons, can also suffer structural alterations upon MBLM binding. In order to test this fact, we examined the effect that MBLM binding has on these protons. Fig. 9 shows the base region of the NOESY spectra of free OL₂ and MBLM-bound OL₂ collected for samples in D₂O at 25 °C. These spectra show that the base protons experience both upfield and downfield shifts upon MBLM binding. The chemical shift differences for the OL₂ base protons after binding to the two Zn(II)BLMs tested are shown in Table 2. Significant shifting (δ greater than or equal to |0.04| ppm) occurred for the C2, A3, G4, T5, T7, T9, T10, C13 and G16 bases for both BLMs studied, although T14 is impacted in the presence of Zn(II)PEP but not in the Zn(II)BLM-A₂ triad. In general, Zn(II)BLM-A₂ causes less of a disturbance (smaller δ s) in the native chemical shifts of the base protons of OL₂ than Zn(II)PEP.

Fig. 10 shows a scheme of the base non-exchangeable protons that experience significant shifts upon MBLM complexation to both OLs. The shifting exhibited by the perturbed base protons in OL₂ is in general greater than that observed in OL₁ [36]. The average $|\delta$ s| observed for OL₁ were 0.03 ppm for the Zn(II)PEP triad and 0.02 ppm for the Zn(II)BLM-A₂. For OL₂ the Zn(II)PEP triad exhibits an average $|\delta|$ of 0.06 ppm and the Zn(II)BLM-A₂ triad shows a value of 0.05 ppm. It is clear from this figure that OL₂ exhibits a higher number of the bases experiencing significant shifting upon Zn(II)BLM-A₂ and Zn(II)PEP binding compared to the number of bases perturbed in OL₁ by the same MBLMs. Upon binding of Zn(II)PEP, 10 out of 16 bases experience a significant shift in OL₂, whereas only 5 out of 14 are affected with OL₁. Binding of Zn(II)BLM-A₂ causes a significant shift for 9 out of 16 bases in OL₂ and 4 out of 14 bases in OL₁. This result also translates into the left strand of OL₂ being more affected by the drugs than the right side, whereas these trends are opposite for OL₁. The possible closeness of the MBLM metal-binding domain to the affected bases in OL₂ could explain the observed δ s, and, if this is the case, OL₂ seems to have closer contact with the MBLMs under study than OL₁.

The number of NOEs connecting non-exchangeable protons detected in free OL₂ and OL₁ that are present in the triads (conserved NOEs) also reinforce this notion. In the OL₂ triads 51% and 49% NOEs are conserved in the left strand of the oligonucleotide in the presence of

Zn(II)PEP and Zn(II)BLM-A₂, respectively. On the other hand, the partner triads formed with OL₁ conserve 92% and 71% of these NOEs [36]. The bases constituting the left strand of OL₁ and OL₂ (Fig. 10) should be more exposed to the influence of the metal-binding domain and linker of BLM. The results discussed above suggest that the metal-binding domain and the linker of the MBLMs have a stronger influence on the bases of the left strand in OL₂ than in OL₁, producing changes in the magnetic and/or chemical environment leading to the δ s and changes of the NOE-network reported herein and in our previous work with OL₁ [36].

The NOEs connecting the base non-exchangeable protons and their corresponding sugar protons (base-sugar NOEs) in OL₂ are also affected as a consequence of MBLM complexation. These NOEs are displayed and labeled in Supplementary Fig. S3, and listed in Supplementary Table S3 for free OL₂. Fig. 11 displays overlays of this region of the spectra for free OL₂ and Zn(II)BLMs-bound OL₂. As it can be seen from Fig. 11, some of the base-sugar NOEs observed in free OL₂ are lost upon MBLM complexation. Furthermore, additional new NOEs are detected connecting the base protons to protons in the corresponding Zn(II)BLM, the corresponding sugar, or the sugar of the previous or next base in the DNA sequence. As concluded in our previous examination of OL₁ complexed to different Zn(II)BLMs [36], each MBLM examined herein affects this region of the NOESY spectra in different ways. On the other hand, in the presence of Zn(II)BLM-A₂ and Zn(II)PEP, OL₁ did not display base-to-base or base-to-Zn(II)BLM NOEs. These results indicate that not only the BLM used, but also the DNA segment considered have an effect on the way the DNA and the MBLM interact with each other.

The data presented in Figs. 9 and 11 has been summarized in schematic representations of free (Supplementary Figure 4) and Zn(II)BLM-bound OL₂ (Fig. 12). These NOEs are listed in Supplementary Table S4. Examination of Supplementary Figure 4 and 12 indicates that the NOEs detected in free OL₂ change differently dependent on the Zn(II)BLM bound. Similar results were found in our studies of various Zn(II)BLMs bound to OL₁ [36], however, the percentage of conserved NOE connections upon MBLM complexation is lower for the MBLM-OL₂ triads. Out of 82 NOEs identified in the D₂O spectra for free OL₂, the Zn(II)PEP-OL₂ triad conserves 66% and the Zn(II)BLM-A₂-OL₂ triad conserves 62 %.

Alternatively, the Zn(II)PEP-OL₁ and Zn(II)BLM-A₂-OL₁ triads conserve 71% and 88% of these NOEs, respectively [36]. As can be inferred from the percentages above, there is a similar degree of the overall change in the external core of OL₂ induced upon binding of the two Zn(II)BLMs examined herein. On the other hand, the changes to the core structure of OL₁ induced by the same Zn(II)BLMs are very different.

Regarding the MBLM binding site in OL₂, comparison of Supplementary Figure S2 and 8 (inter-strand NOEs), and Supplementary Figure 4 and 12 (external core NOEs) shows that the sequential and inter-stand connections between the T11-A12 and A6-T5 bases are greatly affected in the triads, which is consistent with the binding of the MBLMs examined in the 5'-GT-3' binding site. Similar results were obtained from our studies of the OL₁ partner triads for the G10-G11 and C5-C4 bases. These results are consistent with the stacking of one of the thiazolium rings between T11 and A12 in OL₂, and G10 and G11 in

OL₁ [36]. However, the degree of disruption observed in the OL₂ triads is higher than that detected for the OL₁ partner triads. This result validates a greater binding affinity of Zn(II)BLMs to a 5'-GT-3' binding site, as determined through the K_d values found in this investigation.

When the MBLMs considered herein are bound to OL₂ intermolecular NOEs are detected. Fig. 13 shows a schematic representation of these NOEs observed both in H₂O at 5 °C and in D₂O at 25 °C for both triads. Table 3 lists these intermolecular NOEs with their corresponding assignments. It is worth noticing that these NOEs differ in number and in the connected protons between the two triads, indicating that the BLM C-terminus has an effect on the interaction between drug and target. Examination of the intermolecular NOEs detected at 25 °C in D₂O for both triads (Table 3) indicates that the PEP triad exhibits five NOEs connecting the C-terminus of this BLM to bases on the right strand of OL₂, whereas only one NOE connecting the BLM-A₂ C-terminus to T14C6H was detected. Additionally the PEP triad exhibits NOEs linking the Hist C4H and Bit C₃H_a protons to OL₂ base protons. NOEs of this nature were not detected for the OL₂-Zn(II)BLM-A₂ triad. NOEs relating the sugar moieties of both MBLMs to some OL₂ bases provide a hint of the orientation of the metal-binding domains of the drugs relative to OL₂. In the OL₂-Zn(II)PEP triad there is a NOE connecting the disaccharide unit of BLM to the T11C6H proton. On the other hand in the OL₂-Zn(II)BLM-A₂ triad the sugar segment is connected to the G16C8H. This result suggests that the orientations of the metal binding domains in the triads oppose each other. NOEs detected at 5 °C in H₂O for the Zn(II)BLM-A₂ triad connecting the A3C2H and G15NH protons to one of the mannose NH₂ protons support the downward orientation of the metal-binding domain of this MBLM relative to that of the PEP triad. Intermolecular NOEs were not detected when the OL₁ partner triads were studied [36]. This outcome supports our previous assessment that the DNA binding site has also an impact on the mode of binding of Zn(II)BLMs to DNA.

DNA-bound Zn(II)PEP interactions also resulted in a NOE between the methyl group in T5 and the Hist C4H proton. This NOE, together with the intermolecular NOEs detected between the sugar segment in PEP and T11, suggest a Hist moiety in close contact with OL₂, and the sugar unit pointing up. On the other hand, the published solution structure of DNA-bound HOO-Co(III)BLM-A₂ reports hydrogen bonding between the pyrimidinyl ring in BLM-A₂ and the guanine of the 5'-GC-3' binding site [20,29,31,34]. No NOEs or hydrogen bonding were reported connecting the sugar unit in BLM-A₂ and the selected DNA segment, which is consistent with the pyrimidinyl moiety in close contact with the oligonucleotide. These two different pictures suggest a rotation of the metal plane in DNA-bound HOO-Co(III)BLM-A₂ relative to Zn(II)PEP. Whether this result is prompted by the kind of BLM used (BLM-A₂ vs. PEP), the metal center (HOO-Co(III) vs. Zn(II)), or the oligonucleotides considered (d(CCAGGCCTGG) vs. OL₂) remains to be shown. Previous NMR studies of DNA-bound MBLMs have not reported interactions between the sugar segment in BLM and the selected oligonucleotides [20,29,34]. However, as described by Goodwin *et al.*, the crystal structure of the Co(III)BLM-B₂ complexed with DNA resulted in hydrogen bonding between DNA bases and the α-D-mannose moiety in the BLM-B₂ [23]. The disaccharide unit is shown pointing down towards the bases located in the 5' and 3' ends of the oligonucleotide. Similar results were found herein for OL₂-bound Zn(II)BLM-

A₂, supporting possible closeness of the sugar segment in MBLM and DNA. The results of our studies on various Zn(II)BLMs complexed to OL₁ [36] and OL₂ (herein) support the notion that both the BLM C-termini and the DNA binding site have an impact on the conformations of both oligonucleotide and drug. These results suggest that a one-to-one comparison between the crystal structure of Co(III)BLM-B₂ complexed with d(ATTAGTTATAACTAAT) (**1**) and d(ATTAGTTAACTAAAT) (**2**) [23] is unreliable. However, when Zn(II)BLM-B₂ was studied bound to OL₁ [36], the shifts of the NMR signals elicited by protons in the G10, G11, C5, and C4 (binding site) indicated that the C-terminal thiazolium ring stacked between G10 and G11, and the N-terminal thiazolium ring partially stacked between C4 and C5 [36]. This binding mode is similar to that observed for Co(III)BLM-B₂ bound to **1** [23]. On the other hand, Goodwin *et al.* studies of Co(III)BLM-B₂ bound to **1** and **2**, resulted in two different modes of intercalation of the bithiazole unit; part of the C-terminal tail of BLM-B₂ intercalated between base pairs in **2**, but not in **1**; and oligonucleotides dissimilarly unwound upon Co(III)BLM-B₂ binding. These results can be interpreted to indicate that, although the binding sites in both **1** and **2** are 5'-GT-3', the differences in the base sequence in both oligonucleotides prompts different conformations of the bound drug. These findings support one of the main points put forth by the research described herein, suggesting that not only the binding site, but also the DNA base sequence has an influence on the final conformation of the MBLM-DNA triads.

The present study has allowed us to provide evidence that both, the C-termini in different MBLMs, and the binding site in DNA have an impact in the interactions between drug and target. Based on these results, it seems plausible that different metal centers (Co(III), Fe(II), Zn(II), etc.) could also contribute to produce structurally different metal-BLM-DNA triads. Studies involving other metals centers are underway in our laboratory to explore their significance in triad structure.

Conclusions

We have examined the effect of Zn(II)PEP and Zn(II)BLM-A₂ on the conformation of a DNA hairpin of sequence 5'-CCAGTATTTTACTGG-3' (OL₂). This study showed that the two different BLMs affected the DNA structure differently, which is consistent with our previous research involving the binding of various Zn(II)BLMs to DNA hairpin, of sequence 5'-AGGCCCTTTGGCCT-3' (OL₁) [36]. Additionally, comparison of the results obtained from triads formed between Zn(II)PEP and Zn(II)BLM-A₂ with OL₂ and OL₁ indicates that the binding site in DNA (5'-GT-3' in OL₂ and 5'-GC-3' in OL₁) has an impact in the mode of interaction between drug and target. The Zn(II)BLMs selected for the research discussed herein seem to interact more closely with OL₂ as indicated by the extensive disturbance of the native structure of this oligonucleotide caused by the drugs, and the presence of intermolecular NOEs. The NMR data provided here shows the necessity of limiting the variations in DNA fragments, BLM C-termini, and metal centers to be considered in future studies. The judicious control of these factors can lead to the intimate understanding of the interaction between metallo-BLMs and their target DNA, which can guide the synthesis and/or isolation of specific BLM analogs with less severe side effects on cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A	adenine
Ala	β -aminoalanine
Bit	bithiazole
BLMs	bleomycins
C	cytosine
1D	one-dimensional
2D	two-dimensional
δ s	differences in chemical shifts
ω	difference in peak width
FID	free induction decay
G	guanine
Gul	α -L-Gulose
Hist	β -hydroxyhistidine
Mann	α -D-Mannose
MBLMs	metallo-bleomycins
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect cross signals
NOESY	nuclear Overhauser effect spectroscopy
OL	oligonucleotide
OL₁	5'-AGCCTTTTGGCCT-3'
OL₂	5'-CCAGTATTTTACTGG-3'
PEP	peplomycin

ppm	parts per million
Pyr	pyrimidinylpropionamide
SDS	sodium dodecyl sulfate
T	thymine
Tails	C-terminus substituents
Thr	threonine
TOCSY	totally correlated spectroscopy
Val	methylvalerate

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Fig. 1.
Structures of BLM-A₂ and PEP

Fig. 2. Imino region of the 1D ^1H -NMR spectra collected for samples in H_2O at 5°C for: Left panel, free OL_2 and OL_2 bound to Zn(II)BLM-A_2 and Zn(II)PEP . Right panel, equivalent spectra for OL_1 [36]. All samples have a 1:1 Zn(II)BLM:DNA molar ratio

Fig. 3.

Schematic comparison of the effects of Zn(II)BLM-A₂ and Zn(II)PEP on the imino and amino protons in OL₂ and OL₁ [36]. Colored DNA bases experience δ greater than or equal to $|0.04|$ ppm upon MBLM complexation

Fig. 4. Aromatic region of the 1D ¹H-NMR spectra for free and OL₂-bound Zn(II)PEP (left panel) and free and OL₂-bound Zn(II)BLM-A₂ (right panel). The spectra were acquired at 25 °C for samples in D₂O. Zn(II)BLM:DNA complexes are at a 1:1 molar ratio. Supplementary Table S1 lists the chemical shift and peak width information for the Bit and Hist signals before and after binding of the MBLMs to the hairpin

Fig. 5. NOESY spectra of Free-OL₂ in H₂O (red) and D₂O (black) acquired at 5 °C. a NH₂ region. b and c imino proton regions. d methyl region above imino signals. The corresponding assignments are listed in Supplementary Table S2

Fig. 6. Overlays of the amino regions of the NOESY spectra for samples in H₂O acquired at 5 °C for free OL₂ (black) and OL₂ bound to each Zn(II)BLM (red). The NOE signals present in the free-OL₂ spectra that are lost upon MBLM complexation are indicated by asterisks (*), and are identified in Supplementary Table S2. NOE signals that are observed between OL₂ protons only in the presence of the corresponding MBLM are labeled with ('), and their corresponding assignments are listed in Supplementary Table S2

Fig. 7. Overlays of the imino regions of the NOESY spectra for samples in H₂O at 5 °C for free OL₂ (black) and MBLM-bound OL₂ (red). Free-OL₂ NOE signals conserved upon MBLM complexation are labeled with the same numbers used in Fig. 5. New NOE signals are indicated with (‘). The assignments of all these signals are listed in Supplementary Table S2

Fig. 8. Schematic representation of the NOEs between the exchangeable protons for OL₂ bound to the MBLMs in H₂O at 5 °C. The conserved (orange) and new (red) NOEs shown here correspond to spectra in Figs. 5, 6 and 7 and are also listed in Supplementary Table S2. Dashed and continuous lines both represent NOEs, and are used together to avoid confusion in busy regions of the scheme

Fig. 10.

Schematic comparison of the effects of Zn(II)BLM-A₂ and Zn(II)PEP on the non-exchangeable base protons in OL₂ and OL₁ [36]. Colored DNA bases experience δ greater than or equal to $|0.04|$ ppm upon MBLM complexation

Fig. 11.

Overlays of the NOESY spectra for samples in D₂O acquired at 25 °C for free OL₂ (black) and Zn(II)BLM-bound OL₂ (red). Shown here are the NOEs between the base non-exchangeable protons and their corresponding sugar protons. NOEs only detected in the presence of MBLMs are marked with primes (').

Fig. 12.
Schematic representation of the NOE connections for OL₂ bound to MBLM in D₂O at 25 °C. Base-base (green), base-sugar (blue), and new (red) NOEs are shown here with lines and listed in Supplementary Table S4. Dashed lines are used to avoid confusion in the busy sectors of the scheme.

Fig. 13. Schematic representation of the intermolecular NOEs detected after MBLM complexation. Corresponding assignments can be found in Table 3. Blue and red lines represent NOEs detected in H₂O at 5 °C and D₂O at 25 °C, respectively

Table 1Differences in chemical shift between the NH₂ and NH protons in free and MBLM-bound OL₂ for samples in H₂O at 5 °C

Assignment	Chemical Shift (F2 (ppm))				
	OL ₂	OL ₂ -Zn(II)PEP	δ^a (PEP)	OL ₂ -Zn(II)BLM-A ₂	δ^a (A ₂)
C1NH _{2b}	7.63	7.61	0.02	7.64	-0.01
C1NH _{2a}	6.75	6.75	0.00	6.77	-0.02
C2NH _{2b}	8.36	8.29	0.07	8.28	0.08
C2NH _{2a}	6.72	6.66	0.06	6.70	0.02
A3NH _{2b}	7.60	7.56	0.04	7.54	0.06
A3NH _{2a}	6.13	6.11	0.02	6.15	-0.03
G4NH	12.48	12.51	-0.03	12.51	-0.02
T5NH	13.25	13.27	-0.02	13.25	0.00
A6NH _{2b}	7.28	<i>b</i> ₋	-	-	-
A6NH _{2a}	6.31	-	-	-	-
T11NH	13.75	13.82	-0.07	13.86	-0.11
A12NH _{2b}	7.60	7.56	0.04	7.54	0.06
A12NH _{2a}	6.13	6.11	0.02	6.15	-0.01
C13NH _{2b}	7.79	7.78	0.01	7.77	0.02
C13NH _{2a}	6.44	6.46	-0.02	6.44	0.00
T14NH	13.83	13.87	-0.04	13.86	-0.02
G15NH	12.75	12.72	0.03	12.71	0.04
G16NH	12.48	12.56	-0.08	12.51	-0.03

^a Calculated as (chemical shift in free OL₂) - (chemical shift in bound OL₂)^b (-) Unassignable upon complexation with OL₂

Table 2

Chemical shifts of the base protons in free and MBLM-bound for samples in D₂O at 25 °C at a 1:1 molar ratio

Chemical Shift (F2 (ppm))						
Base	¹ H	OL ₂	OL ₂ -PEP ^a	δ ₀ (PEP)	OL ₂ -A ₂ ^a	δ ^b (A ₂)
C1	(C6H)	7.70	7.71	0.01	7.71	0.01
C2	(C6H)	7.53	7.43	0.10	7.46	0.07
A3	(C8H)	8.20	8.03	0.17	8.08	0.12
G4	(C8H)	7.59	7.64	-0.05	7.64	-0.04
T5	(C6H)	7.00	6.90	0.10	6.93	0.07
A6	(C8H)	8.18	8.17	0.01	8.20	-0.02
T7	(C6H)	7.18	7.25	-0.07	7.27	-0.08
T8	(C6H)	7.39	7.39	0.00	7.39	0.00
T9	(C6H)	7.44	7.39	0.05	7.39	0.05
T10	(C6H)	7.49	7.30	0.19	7.39	0.10
T11	(C6H)	7.47	7.50	-0.03	7.47	0.00
A12	(C8H)	8.30	8.32	-0.02	8.31	0.01
C13	(C6H)	7.35	7.44	-0.09	7.41	-0.06
T14	(C6H)	7.25	7.20	0.05	7.22	0.03
G15	(C8H)	7.78	7.79	-0.01	7.78	0.00
G16	(C8H)	7.76	7.68	0.08	7.70	0.06

^aChemical shift of the proton in OL₂ when bound to the corresponding Zn(II)BLM.

^bCalculated as (chemical shift in free OL₂) – (chemical shift in bound OL₂).

Table 3Intermolecular NOEs detected between OL₂ and MBLMs protons

Assignment	F2 (ppm)	F1 (ppm)	OL ₂ -Zn(II)PEP	OL ₂ -Zn(II)BLM-A ₂
^a A12C8H-PEP C-terminus C _α H _β	8.31	2.87	+ ^c	
^a T10C6H-PEP C-terminus C _δ CH ₃	7.30	1.58	+	
^a T10C6H-PEP C-terminus C _α H _α	7.30	2.74	+	
^a T10C6H-PEP C-terminus C _α H _β	7.29	2.88	+	
^a T11C6H-PEP C-terminus C _α H _α	7.50	2.75	+	
^a T5 CH ₃ -Hist C4H	1.37	7.33	+	
^a A12C8H-Bit C _α H _α	8.33	3.37	+	
^a T11C6H - Zn(II)BLM sugar	7.51	4.92	+	
^a G16C8H-Zn(II)BLM sugar	7.71	3.68		+
^a T14C6H - A ₂ C-terminus C _β H ₂	7.22	3.31		+
^b A12/A3C2H - Mann NH _{2b}	7.37	6.31		+
^b G15NH-Mann NH _{2b}	6.28	12.75		+

^a Detected in D₂O at 25 °C.^b Detected in H₂O at 5 °C.^c Plus (+) indicates that the specific NOE connection is found upon complexation with the specified Zn(II)BLM.