Published in final edited form as:

J Biol Inorg Chem. 2017 January ; 22(1): 121–136. doi:10.1007/s00775-016-1413-4.

NMR Study of the Effects of Some Bleomycin C-termini on the structure of a DNA Hairpin with the 5'-GC-3' Binding Site

Teresa E. Lehmann[✉], Sally A. Murray, Azure D. Ingersoll, Teresa M. Reilly, Shelby E. Follett, Kevin E. Macartney, and Mark H. Harpster

Department of Chemistry, University of Wyoming, 1000 E. University Avenue, Laramie, Wyoming 82071, US

Abstract

The antibiotics known as bleomycins constitute a family of natural products clinically employed for the treatment of a wide spectrum of cancers. The drug acts as an antitumor agent by virtue of the ability of a metal complex of the antibiotic to cleave DNA. Bleomycins are differentiated by their C-terminal regions. Previous structural studies involving metal-bleomycin-DNA triads have allowed the identification of the bithiazole-(C-terminus substituent) segment in this molecule as the one that most closely interacts with DNA. Three different modes of binding of metallo-bleomycins to DNA (partial or total intercalation of the bithiazole unit between DNA bases, or binding to the minor groove) have been proposed in the literature. The therapeutic use of bleomycin is frequently associated with the development of pulmonary fibrosis. The severity of this side effect has been attributed to the C-terminus of the antibiotic by some researchers. The degree of pulmonary toxicity of bleomycin-A₂ and -A₅, were found to be higher than those of bleomycin-B₂ and peplomycin. Since the introduction of Bleomoxane to clinical medicine in 1972, attempts have been made at modifying the basic bleomycin structure at the C-terminus to improve its therapeutic index. However, the pharmacological and toxicological importance of particular C-termini on bleomycin remains unclear. The present study was designed to determine the effect of Zn(II)bleomycin-A₂, -A₅, -B₂, and Zn(II)peplomycin on the structure of a DNA hairpin containing the 5'-GC-3' binding site. We provide evidence that different Zn(II)bleomycins affect the structure of the tested DNA segment in different fashions.

Keywords

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

Introduction

The antitumor antibiotics known as bleomycins (BLMs) (Fig. 1) are complex glycopeptides isolated from cultures of *Streptomyces verticillus* [1]. Clinically, BLMs are employed for the treatment of squamous cell carcinomas [2], non-Hodgkin's lymphomas [3], testicular carcinomas [4], and ovarian cancer [5]. The drug acts as an antitumor agent by virtue of the ability of a metal complex of the antibiotic to cleave DNA [6]. The BLM structure can be

[✉] tlehmann@uwyo.edu. Fax: (307) 766-2807.

separated into a metal-binding domain, which provides the ligands for metal complexation, a tumor-targeting disaccharide domain [7]; and a DNA-binding domain, composed of a bithiazole moiety attached to a C-terminus substituent (Fig. 1). BLM congeners are differentiated by this unique amine substituted C-terminal region. Previous structural studies involving metallo-bleomycins (MBLMs) and MBLM-DNA triads have identified the bithiazole-(C-terminus substituent) segment in this molecule as the one that more closely interacts with DNA [8–19]. However, the metal-binding domain has also been found to have an influence on the DNA-binding strength and specificity of the antibiotic. To date, the structures of MBLM-DNA complexes published in the literature [8–19] have been determined using different MBLMs, interacting with different DNA fragments, with a focus on the intercalating capability of the bithiazole-segment and a disregard of the effect of the substituent C-termini upon binding. The results of these studies have led to the proposal of three different modes of binding of MBLMs to DNA (i.e. partial [13,15] or total [19] intercalation of the bithiazole unit between DNA bases, or binding to the minor groove [11]), which have created controversy in the field, and uncertainty regarding whether the MBLM-DNA structures that have been characterized approximate the structures that are assembled *in vivo*. In summary, the absence of a systematic approach to controlling experimental factors such as DNA-base sequences, metal centers, and C-terminus substituents (tails) in the aforementioned studies have led to a lack of clarity when it comes to characterizing the solution structure of these drugs, and their authentic interactions with DNA. Methodical studies controlling each of these factors individually are required in order to establish their relative significance.

Notwithstanding the absence of a coherent model for the interaction of BLMs with DNA, the mechanism by which the therapeutic use of BLM is frequently associated with the development of pulmonary fibrosis is poorly understood. However, rodent models developed for the study of pulmonary fibrosis and metaplasia upon BLM administration [20–30] have indicated that the severity of BLM-induced lung injury in animals can be attributed to the tails which differentiate BLMs. The degree of pulmonary toxicity of BLM-A₂ and -A₅, for instance, were found to be higher than those of BLM-B₂ and peplomycin (PEP). On the other hand, correlations between lung-cell toxicity and anticancer activity by BLM are more tenuous. For instance, PEP has been found to exhibit stronger anticancer activity, with lower pulmonary toxicity [23, 24]. CuBLM-A₂ has been reported having stronger antitumor activity than CuBLM-B₂ against Ehrlich carcinoma and sarcoma 180 [31]. Phleomycin obtained from the culture of filtrate of *Streptomyces verticillus* and separated into various compounds (A, B, C, D₁, D₂, E, F, G, H, I, J, and K) were found to exhibit different degrees of antibacterial, anticancer, and lung-damaging activities [32]. The presence of a metal center has also been characterized as an essential step for the anticancer activity attributed to BLM. However, copper complexes of BLM have produced pulmonary disease comparable to their respective metal-free BLM and phleomycin analogs when administered to DBA/2J mice by endotracheal route [21]. Extensive research into less toxic BLMs has produced 300 additional C-terminal-tail analogs that have been screened for increased antitumor activity and decreased pulmonary cytotoxicity [23,24,29,30]. In the medical field, significant research efforts are devoted each year toward eliminating or alleviating pulmonary fibrosis caused by BLM in cancer patients through combination chemotherapy. Although some of

these efforts have produced successful outcomes, the results are not general enough to be applied to a wide cancer spectrum [33]. The connection between the BLM tails and pulmonary fibrosis established by Raisfeld *et al.* [20–22, 25–28] has supported the development of new BLMs with less toxic tails. Additionally, since the introduction of the clinically used mixture of BLMs, Blenoxane, to clinical medicine in 1972, attempts have been made at modifying the basic BLM structure at the C-terminus to improve its therapeutic index. However, other than their demonstrated role in binding to DNA, the pharmacological and toxicological importance of particular tails on BLM remains unclear.

Since the C-termini of BLM-A₂, -A₅, -B₂, and PEP have different chemical structures, it is plausible to think that they could produce different conformations of their corresponding MBLM-DNA triads. The present study was designed to determine the effect of four different Zn(II)BLMs (Zn(II)BLM-A₂, -A₅, -B₂, and PEP) on the structure of a DNA hairpin of sequence 5'-AGGCCTTTTGGCCT-3', containing the 5'-GC-3' binding site. The selected metal center 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 [12] are the same found by us in our extensive studies of Fe(II)BLM complexes [34–39]. This is the first on a series of studies of MBLMs-DNA interactions that we plan to conduct, aimed to determine how some factors such as: metal centers, DNA-binding sites, and BLM C-termini groups, influence the structures of MBLM-DNA triads. We provide evidence that different Zn(II)BLMs affect the structure of the tested DNA segment in different ways.

Materials and methods

Materials

BLM-A₂ and -B₂ sulfate were purchased from TOKU-E (Bellingham, WA). BLM-A₅ was purchased from LKT Laboratories, Inc. (St Paul, MN). 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 oligonucleotide was purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

NMR Sample Preparation

BLMs, 1.95 μmol , were dissolved in 650 μL of D₂O. A 0.12 M solution of ZnSO₄•7H₂O was then added to the BLM solutions to afford Zn(II):BLM mole ratios of 1:1. The pH (meter reading uncorrected for the deuterium isotope effect) was adjusted to 6.5 with a 0.1 M NaOD solution. DNA, 0.325 μmol , was dissolved in 585 μL of D₂O. Subsequently, 65 μL of a 200 mM NaCl solution was added, and the pH was adjusted to 6.5. Various aliquots of Zn(II)BLM were then added to the DNA sample and the Zn(II)BLM:DNA 1:1 complex formation was followed by monitoring the changes in one-dimensional (1D) ¹H-NMR spectra. When the DNA to Zn(II)BLM ratio becomes 1:1, no additional changes are observed in the 1D spectra of the samples. All Zn(II)BLM-DNA samples were prepared at

Zn(II)BLM:DNA molar ratios 1:1. Zn(II)BLM samples in 90% H₂O/10% D₂O (referred to as spectra in H₂O) were prepared by analogous procedures.

NMR Spectra Collection

NMR experiments were performed at 600 MHz on a Bruker AVANCE III 600 spectrometer (Bruker BioSpin Corp, Billerica, MA) with a 5.0 mm multi-nuclear broad-band observe probe. Spectra were collected at 278 MK (samples in H₂O), and 298 K (samples in D₂O), and referenced to HDO and H₂O as the internal standards. For the two-dimensional total correlation spectroscopy (TOCSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY), experiments, solvent suppression was achieved using excitation sculpting with gradients. The mixing times in NOESY and TOCSY experiments were typically 200 ms and 40 ms, respectively. Spectral widths were typically 10 ppm (samples in D₂O), and 18 ppm (samples in H₂O) 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 was usually 32. Spectra were Fourier transformed using Lorentzian-to-Gaussian weighting and phase-shifted sine-bell window functions. Processing and analysis of the NMR data were performed using Topspin3.0 and NMRViewJ software.

Results

Fig. 2 shows the imino region of the 1D ¹H-NMR spectra in H₂O collected for the oligonucleotide (OL) both in the absence and presence of Zn(II)BLM-A₂, -A₅, -B₂, and Zn(II)PEP. The broadening and shifting of the imino signals in OL is an indication of the drugs' binding to OL [10]. However, it is clear that not all of the BLMs tested affect the imino region in the same fashion. Binding of Zn(II)BLM-A₂ and -B₂ to OL, for instance, produce similar shifting, with more significant broadening exhibited in the presence of Zn(II)BLM-B₂. On the other hand, binding of Zn(II)BLM-A₅ to OL produces the most significant broadening of the imino signals in OL. Finally, binding of Zn(II)PEP to OL causes broadening, but only slight shifting of the OL imino signals.

The bithiazole (Bit) and β-hydroxyhistidine (Hist) signals in each Zn(II)BLM exhibit changes upon complexation to OL. Fig. 3 shows the aromatic region of 1D ¹H-NMR spectra collected for each Zn(II)-bound antibiotic free and complexed with OL in D₂O at 25 °C.

In all cases, there is broadening and shifting of the aromatic signals of these moieties for each Zn(II)-BLM-OL triad indicating binding to OL. However, there are differences in their behavior depending on the Zn(II)BLM bound (Supplementary Table S1). Zn(II)BLM-A₂-OL and -B₂-OL exhibit similar degrees of upfield shifting of the Hist signals, with more shifting of the Bit in Zn(II)BLM-A₂-OL. Zn(II)BLM-A₅-OL displays intense broadening and downfield shifting of the Bit signals. More broadening of the Hist signals than in Zn(II)-BLM-A₂-OL and -B₂-OL is also observed. For Zn(II)PEP-OL the Bit signals are sharper and less upfield shifted than for the other triads. Both of the Hist signals in Zn(II)BLM-B₂-OL are sharp, while one of these signals is broadened to a larger extent for Zn(II)BLM-A₂-OL. The Hist signals identified in the spectra of Zn(II)BLM-A₅ and Zn(II)PEP bound to OL are broadened. Interestingly, the ¹H signals elicited by the ring present in the C-terminus of PEP exhibit broadening upon complexation to OL, but no significant shifting.

NOESY spectra in H₂O of free OL and OL in the presence of the MBLMs under study were also collected. These data were acquired at 5 °C to allow unambiguous assignments of the exchangeable NH and NH₂ protons in OL. The NH and NH₂ signals in OL have been labeled in Fig. 4, and the assignments are shown in Supplementary Table S2.

Fig. 4 shows an overlay of the NOESY spectra collected for OL at 5 °C in H₂O (red) and D₂O (black). Overlays of the 5 °C NOESY spectra in H₂O collected for free OL (black) and OL bound to BLM-A₂, -A₅, -B₂ and PEP (red) are shown in Fig. 5. This figure shows how some of the NH and NH₂ signals in the bound OL exhibit shifting from their positions in free OL.

Table 1 displays the difference in chemical shifts (δ s) of these signals when compared with those of the free OL (Fig. 5 and Supplementary Table S2). For all bound BLMs there is significant shift (δ bigger than or equal to |0.04|) of one of the protons in the C5NH₂ and C13NH₂ groups in OL, while the other NH₂ protons exhibit from moderate to very small shifts. Interestingly, the binding of Zn(II)PEP to OL produces the lowest degree of disturbance of the NH₂ signals of OL, while Zn(II)BLM-A₂ and -B₂, and -A₅ cause similar degrees of disturbance of these signals.

Regarding the imino signals (Fig. 6, NOESY signals labeled based on the numbering in Fig. 4B). Table 1 also shows that, in general, G10 and G11 display the most significant shifts of these NH signals. A more detailed comparison of the δ s obtained for the imino signals indicated that, as shown for the NH₂ peaks, the binding of Zn(II)PEP produces the least disturbance of the NMR signals.

As can be seen in Fig. 5, some of the non-imino NOEs detected for free OL in H₂O are missing in the presence of the antibiotics (indicated by arrows in Fig. 5, and compiled in Supplementary Table S3). Some of the missing NOEs are common to all bound OL, but there are also differences in the missing NOEs depending on the bound MBLM. The data presented in Figs. 5 and 6 has been summarized in schematic representations of free (Fig. 7) and Zn(II)BLM-bound OL (Fig. 8) for easy visualization. Zn(II)BLMs have been depicted binding OL as suggested by Wu et al. [13].

Fig. 7 displays the 53 NOEs detected for free OL in H₂O at 5 °C, and Fig. 8 shows the NOEs displayed by OL that are conserved when a particular Zn(II)BLM is bound (no new NOEs between protons in OL were detected in the spectra of OL bound to the MBLMs). Examination of Fig. 8 shows that many of the conserved NOEs are all on the plane of the base pairs, which indicates that these planes are not disturbed upon Zn(II)BLM binding. Careful analysis of Figs. 5 and 6 and Supplementary Table S3, which account for the missing NOEs upon Zn(II)BLM binding, indicates that from a total of 53 NOEs detected for free OL in H₂O (Supplementary Table S2), 64% are conserved after Zn(II)PEP binds OL, while 49%, 45%, and 43% are conserved upon Zn(II)BLM-A₅, -B₂, and -A₂ complexation, respectively. From these results, it can be proposed that Zn(II)PEP disturbs the NOE network present in OL to a lesser extent than the other BLMs. An interesting result that arises from this comparison is that almost all the NOEs conserved in the presence of Zn(II)BLM-B₂ are also conserved upon Zn(II)PEP complexation.

The manner in which the DNA bases shift in the NMR spectra collected upon complexation with different Zn(II)-bound antibiotics is also worth examining. Fig. 9A shows part of the base region of the NOESY spectra collected at 25 °C in D₂O for free OL (black) and OL in the presence of Zn(II)BLM-A₂, -A₅, -B₂, and PEP (red). It is clear from this figure that the degree and direction (upfield or downfield) of shifting of the base signals varies upon complexation of different BLMs to OL. Table 2 presents the chemical shifts of the signals depicted in Fig. 9A for each OL-bound Zn(II)BLM and free OL. Differences in chemical shifts between the free and Zn(II)BLM-bound OL are also included. As can be concluded from examination of Table 2, binding of the MBLMs to OL causes significant downfield shifts of G10 and moderate downfield shifting of G2 and T9, albeit to varying degrees for each bound MBLM. Binding of Zn(II)BLM-A₅, -B₂, and Zn(II)PEP to OL induces upfield or downfield shifting of additional bases.

The information gathered in Tables 1 and 2 was summarized in schematic representations showing the Zn(II)BLMs bound to OL as proposed by Wu and coworkers [13] (Fig. 9B). Examination of Fig. 9B shows that the binding of each MBLM produces significant shifting of the C5, G10, T9, G11, G2, and C13 bases. These are the only bases affected upon Zn(II)BLM-A₂ binding to OL, while binding of the other MBLMs affects additional bases.

The DNA bases in OL exhibit NOESY connectivities with neighboring bases and their sugar units. As an example, some of these connectivities are shown in Supplementary Fig. S1, and are compiled in Supplementary Table S4. We examined the effect of the different Zn(II)BLMs on these NOEs. Fig. 10 displays overlays of the NOESY spectra collected for free OL (black) and MBLM-bound OL (red) at 25 °C in D₂O. As can be seen from this figure, in addition to the shifting of the bases already mentioned, some of the inter-base base-sugar (b-s) NOEs detected for free OL are absent in MBLM-bound OL. This data is compiled in Supplementary Table S5. Global analysis of the NOESY spectra collected in D₂O at 25 °C indicated that also some of the inter-base base-base (b-b) NOEs between OL protons are disturbed upon binding of the examined MBLMs.

The bulk and complicated nature of the spectra generated in this investigation makes it very difficult to present them all. However, schematic representations of OL displaying all inter-base NOEs in its free and bound forms are presented in Figs. 11 and 12, in order to facilitate the visualization of the effect the Zn(II)BLMs have on OL. Supplementary Table S6 compiles the differences in the b-s and b-b connections in the NOESY spectra of free and bound OL.

Examination of Figs 11 and 12 indicates the BLM-A₅ triad conserves the lowest number of original OL NOEs (68%), while the BLM-A₂ triad has 88% of these NOEs, with the BLM-B₂ and PEP triads exhibiting 73% and 71%, respectively.

Discussion

The data presented on Fig. 2 indicate that, in general, all the MBLMs under study bind OL, since the imino signals in the oligonucleotide are broadened and shifted upon the triad formation. However, DNA-intercalators generally produce upfield shifting of the DNA-

imino ^1H signals [40]. What we observe in the present study is downfield shifting of these signals, which is more in agreement with binding of the drugs in a groove of OL [40]. Additional support to this mode of binding is provided by the unfused nature of the Bit ring system [40]. The slight differences recorded in Fig. 2 upon the binding of the studied Zn(II)BLMs are clearly caused by the different BLM tails, with the PEP and BLM-A₅ triads exhibiting the lowest and highest degrees of disturbance, respectively.

Shifting and broadening of the Hist ring signals was reported for Zn(II)BLM-A₅ bound to d(CGCTAGCG)₂ [11], and attributed to interaction of the Hist 2 proton with the edges of the DNA base pairs. In the present study, the effect of OL on these signals is diverse (Fig. 3), indicating that the interaction of the Hist moiety with the DNA base pairs is modified according to the BLM C-terminus. Based on the spectra shown in Fig. 3 and the results displayed in Supplementary Table S1, it seems that there is little interaction of Hist with OL in Zn(II)BLM-B₂, moderate interaction in Zn(II)BLM-A₂, and strong interaction in Zn(II)BLM-A₅ and Zn(II)PEP. Interestingly, the bulky tail present in PEP does not appear to be greatly affected by binding to OL, which would indicate a low degree of interaction with OL, as reported by Caceres-Cortes *et al.* for HO-Co(III)PEP [8].

As indicated in Tables 1, 2 and Fig. 9B, the NOESY spectra of OL in the presence of Zn(II)BLMs exhibit shifts of some of the proton signals of the oligonucleotide. Shifts of the signals elicited by the protons in C5, G10 and G11 are expected, since those bases form the BLM binding site. Significant shifts of these signals are consistent with stacking of the terminal thiazolium ring between the bases of G10 and G11, and the penultimate thiazolium ring partially stacked between the bases of C5 and C4, as reported by Wu *et al.* for HO-Co(III)BLM-A₂ [13]. Our results indicate that this is the case for Zn(II)BLM-A₂ and -B₂. However, in the presence of Zn(II)BLM-B₂ the imino signal of G3 shows an extra shift of -0.05 ppm, possibly caused by a distortion of the OL structure upon binding to this BLM. Additionally, the binding of Zn(II)BLM-B₂ affects the hairpin loop more extensively, as indicated by the shifts exhibited by T6. These effects are absent in the BLM-A₂ triad. Using the same set of arguments for the BLM-A₅ triad could indicate stacking of the terminal thiazolium ring between the bases of G10 and G11, and stacking of the penultimate thiazolium ring between the bases of C5 and C4. This type of binding has been reported for HO-Co(III)BLM-B₂ [19]. The shifts exhibited by the C13NH₂ hydrogens could be interpreted in two different ways. First, there could exist a second minor binding site between C13G2 and C12G3. However, the lack of significant shifts of G2 and A1 or G3 and C12 do not support this argument. On the other hand, the binding of the MBLMs to OL could cause distortions on the OL structure that would locate the C13 and G2 protons in a different chemical or magnetic environment, producing the aforementioned shifts. Distortions (bends) of the structures of oligonucleotides bound to Zn(II)BLMs have previously been reported [11].

Upon Zn(II)BLM complexation to OL, some of the NOEs detected in H₂O and D₂O for free OL disappear indicating that the distances between the protons generating those NOEs becomes more than 5 Å. Additionally new NOE connectivities arise as a consequence of MBLM binding (Figs. 8 and 12, and Supplementary Tables S3, S5, and S6). Regarding the missing NOEs, it is better to separate the results shown in Fig. 8 from those displayed in Fig.

12, since the NOEs detected in H₂O for the NH and NH₂ protons describe the interaction between base pairs, whereas the NOEs detected in D₂O describe the changes in the exterior core of OL. A comparison of Fig. 7 and 8 clearly shows that many of the NOEs connecting the G10C5 to G11C4 and C12G3 to C13G2 base pairs are absent from OL after Zn(II)BLM complexation. These results are consistent with changes in the structure of OL upon MBLM binding between G10C5 and G11C4, and a possible distortion of the OL structure in the C12G3-C13G2 region. Of paramount importance are the imino NOEs (numbers 38, 39, and 45, Fig. 4B), which connect the G10C5 and G11C4 base pairs. These NOEs are absent in bound OL and thereby support a model of Zn(II)BLM binding between these base pairs for all triads. In the PEP and BLM-A₅ triads, more NOEs are conserved in OL upon binding. This is possibly an indication of weaker binding by these MBLM, which would render a less disturbed average structure for OL. Outside of these finding, it is difficult to perform a detailed comparison between triads, since each one exhibits a different situation regarding the conserved NOEs. As the different MBLMs are binding to the same OL, and the difference between BLMs resides in the C-termini, it is possible to suggest that the BLM tails have an influence in the positioning of BLM around its binding site in OL.

Examination of Fig. 12 allows us to state that the network of core-NOE connectivities displayed by free OL changes differently upon the binding of the various MBLMs. In particular, the NOE connectivities in the T9-G10-G11 string of bases is strongly affected for each MBLM, consequent to the formation of all triads. This result is in agreement with changes in the structure of the OL core if the terminal thiazolium ring is stacked between the bases of G10 and G11. In contrast, the conformation of the T6-C5-C4 string is disparate, possibly indicating different extents of partial or total intercalation of the penultimate thiazolium ring between the bases of C5 and C4. It is also possible that the metal-binding domain in BLM is positioned in different fashions in the vicinity of the left stand of bases of OL upon complexation. Different conformations of the metal-binding domain of BLM when bound to a wide variety of DNA segments have been proposed in a plethora of scientific publications reporting MBLM-DNA interactions.

Global examination of the NOEs detected for free and bound OL indicates that Zn(II)PEP seems to affect the OL structure to less of an extent than the other BLMs examined in this study. Zn(II)BLM-A₅ either has a great effect on the core of the hairpin, or there are multiple modes of binding of this particular BLM to OL resulting on an average structure assessed through NMR that only shows the NOEs conserved for all possible conformations after different modes of binding.

Zn(II)BLM interactions with the oligonucleotide d(CGCTAGCG)₂ have been studied previously, and a solution structure of this triad has been reported [12]. The required NMR data to generate this structure involved only eight NOE signals connecting the MBLM with the chosen DNA fragment, which were detected only when the NOESY spectra were collected at 35 °C. The NOESY data reported in the present study were collected at either 25 or 5 °C, and no intermolecular NOEs were observed at these temperatures to allow molecular dynamics calculation leading to solution structures of the considered triads. However, the NMR data presented herein allowed the assignments of all the ¹H signals in the chosen hairpin, including -NH and -NH₂ groups. Many of this signals were not assigned

in the previously reported study of Zn(II)BLM-DNA [12], possibly due to the high temperature used, and they are of paramount importance to determine the imino/amino behavior of the DNA segment studied. Although a structure of the MBLM-DNA triads did not result from the present study, the δ s provided are a strong sign of the different chemical/magnetic environments experienced by the hairpin when bound to different Zn(II)BLMs. These δ s indicate different conformations of the hairpin that can only be prompted by the BLM C-termini. Another feature of the Zn(II)BLM-DNA interaction pointed out by Manderville *et al.* [12] is that Zn(II)BLM is in fast exchange with d(CGCTAGCG)₂. Fast exchange is concluded by these researchers based on the fact that the strands of the self-complementary oligonucleotide remain equivalent upon Zn(II)BLM-A₅ binding. The NMR data collected for this work indicate an intermediate exchange rate at 25 °C. This fact is evinced by the diminishing in intensity of some of the NMR signals generated by free OL, as aliquots of the Zn(II)BLMs are added during the titration experiments described in Materials and methods. Additionally, new signals are observed when the Zn(II)BLM:DNA ratio reaches 1:1. Similar results were reported by Wu *et al.* during studies of the binding of Ho-Co(II)BLM to a DNA fragment [14], and interpreted as a slow exchange regime. An example of our titration experiments is provided in Supplementary Figure S2. It is possible that the binding of Zn(II)BLM to d(CGCTAGCG)₂ is weak, and at high temperatures (35 °C) the MBLM could enter a fast-exchange regime with the oligonucleotide.

The coordination geometries and solution structures of MBLMs have been strongly linked to the bound metal center. Based on this fact, it is conceivable to consider the possibility that the results presented herein might change if other metal centers (Co, Fe) are bound to BLM. Additionally, activation of MBLMs involves the binding of –OOH to the metal center, and it could lead to changes on the binding of the activated drug to DNA. Work is underway in our lab to explore these possibilities.

Although the source of BLM pulmonary toxicity has not been linked to its interaction with DNA, it is interesting to notice that PEP and BLM-A₅ are at the opposite ends of the spectrum of pulmonary toxicities [20–30] displayed by this family of antibiotics. Based on the results of the present study, it is tempting to propose a possible connection between the level of disturbance of DNA upon MBLM complexation, and that of pulmonary toxicity resulting from the use of different BLMs in cancer chemotherapy. Detailed structural characterization at the molecular level of BLM binding to DNA and the structure/toxicity connection could unveil even more intimate details of the MBLM-DNA triads, which could advance the BLM field vertically by guiding the synthesis and/or isolation of specific congeners with tails producing very low or no pulmonary toxicity. These new BLMs could improve the quality of life of cancer patients under BLM regimens. The development of more intensive chemotherapy regimens has increased the incidence of toxic pulmonary side effects following administration of an increasing number of commonly used cytotoxic drugs [41–44], and pulmonary complications are no longer restricted to BLM. It is possible that the structure/toxicity correlations to be delineated as a result of detailed studies of MBLM-DNA interactions will help to better understand this side effect when caused by other drugs.

The binding of BLMs to DNA has been studied extensively, however, there is no experimental evidence to date allowing an unequivocal identification of the DNA-binding mode characteristic of metallo-BLMs (MBLMs). This lack of consensus is generated mainly because BLM binding to DNA depends on the DNA base sequence, the metal center and its activation state, and the chemical structure of the tail fragments. BLM tails are important components of both DNA binding and pulmonary toxicity. Therefore, a thorough understanding of the structural interactions between BLM and DNA is necessary to settle the controversy involving BLM binding to DNA, and to establish structure/toxicity correlations that can help guide the development of new BLM congeners to reduce the burden of illness caused by cancer.

Conclusions

We have examined the effects of different Zn(II)BLMs on the conformation of a DNA hairpin with the sequence 5'-AGGCCTTTTGGCCT-3'. This study introduces a level of consistency and comparability currently missing in the BLM research that has been published to date. The findings of the present study indicate that the BLM C-termini have a profound effect on the resulting structure of the target oligonucleotide when bound to Zn(II)BLM. Additionally, the degree of partial or total intercalation of the bithiazole moiety between the oligonucleotide base pairs also seems to be impacted by the BLM tails. The data collected in the research described herein is currently being examined to investigate potential changes in BLM structure as a consequence of DNA binding. This involves determining how the NMR signals common to Zn(II)BLM-A₂, -A₅, -B₂, and Zn(II)PEP, mainly metal-binding domain, bithiazole, and linker, are affected upon complexation to OL. Additionally, studies on the binding of the Zn(II)BLMs used in the present study to DNA hairpins containing the 5'-GT-3' binding sites are also underway in our lab to determine possible differences between the reported preferential binding sites of BLM to DNA. The pulmonary toxicity and DNA interactions of MBLMs have not been directly linked to date, and MBLM toxicity towards cells in general has been attributed to high concentrations of the drug in healthy and diseased tissues. We are designing studies to approach the possible correlation between these two features of MBLMs through studies of binding of MBLMs to carefully selected RNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in whole by the National Institute of Health [Grant 1R15GM106285-01A1]. Our gratitude also goes to Nippon Kayaku Co., Ltd. (Tokyo, Japan) for the generous gift of peplomycin. We also acknowledge Dr. Alexander Goroncy for help collecting the NMR data presented in this work.

Abbreviations

A	adenine
Ala	β-aminoalanine

b-b	base-base
Bit	bithiazole
BLMs	bleomycins
b-s	base-sugar
C	cytosine
1D	one-dimensional
δs	differences in chemical shifts
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
PEP	peplomycin
ppm	parts per million
Pyr	pyrimidinylpropionamide
s-s	sugar-sugar
T	thymine
Tails	C-terminus substituents
Thre	threonine
TOCSY	totally correlated spectroscopy
Val	methylvalerate

References

1. Umezawa H, Maeda K, Takeuchi T, Okami J. *J Antibiot.* 1966; 19:200–209. [PubMed: 5953301]
2. Bennett JM, Reich SD. *Ann Intern Med.* 1979; 90:945–948. [PubMed: 87142]

3. Carlson RW, Sikic BI, Turbow MM, Ballon SC. *J Clin Oncol.* 1983; 1:645–651. [PubMed: 6199468]
4. Hecht, SM. *Cancer Chemotherapeutic Agents*. Foye, WO., editor. ACS Professional Reference Book; Washington, D. C: 1995. p. 369-388.
5. Einhorn LH, Donohue J. *Ann Intern Med.* 1977; 87:293–298. [PubMed: 71004]
6. Umezawa, H. *Bleomycin: chemical, biochemical and biological aspects*. Hecht, SM., editor. Springer; New York: 1997. p. 24-36.
7. Schroeder BR, Ghare MI, Bhattacharya C, Paul R, Yu ZQ, Zaleski PA, Bozeman TC, Rishel MJ, Hecht SM. *J Am Chem Soc.* 2014; 136:13641–13656. [PubMed: 25184545]
8. Caceres-Cortes J, Sugiyama H, Ikudome K, Saito I, Wang AHJ. *Biochemistry.* 1997; 36:9995–10005. [PubMed: 9254594]
9. Hoehn ST, Junker HD, Bunt RC, Turner CJ, Stubbe J. *Biochemistry.* 2001; 40:5894–5905. [PubMed: 11352724]
10. Lui SM, Vanderwall DE, Wu W, Tang X-J, Turner CJ, Kozarich JW, Stubbe J. *J Am Chem Soc.* 1997; 119:9603–9613.
11. Manderville RA, Ellena JF, Hecht SM. *J Am Chem Soc.* 1995; 117:7891–7903.
12. Vanderwall DE, Lui SM, Turner CJ, Kozarich JW, Stubbe J. *Chem Biol.* 1997; 4:373–387. [PubMed: 9195876]
13. Wu W, Vanderwall DE, Stubbe J, Kozarich JW, Turner CJ. *J Am Chem Soc.* 1994; 116:10843–10844.
14. Wu W, Vanderwall DE, Lui SM, Tang X-J, Turner CJ, Kozarich JW, Stubbe J. *J Am Chem Soc.* 1996; 118:1268–1280.
15. Wu W, Vanderwall DE, Turner CJ, Kozarich JW, Stubbe J. *J Am Chem Soc.* 1996; 118:1281–1294.
16. Wu W, Vanderwall DE, Teramoto S, Lui SM, Hoehn ST, Tang X-J, Turner CJ, Boger DL, Kozarich JW, Stubbe J. *J Am Chem Soc.* 1998; 120:2239–2250.
17. Wu W, Vanderwall DE, Turner CJ, Hoehn S, Chen JY, Kozarich JW, Stubbe J. *Nucleic Acids Res.* 2002; 30:4881–4891. [PubMed: 12433991]
18. Zhao CQ, Xia CW, Mao QK, Forsterling, DeRose E, Antholine WE, Subczynski WK, Petering DH. *J Inorg Biochem.* 2002; 91:259–268. [PubMed: 12121784]
19. Goodwin KD, Lewis MA, Long EC, Georgiadis MM. *Proc Nat Acad Sci USA.* 2008; 105:5052–5056. [PubMed: 18362349]
20. Raisfeld IH, Chovan JP, Frost S. *Life Sci.* 1982; 30:1391–1398. [PubMed: 6177995]
21. Raisfeld IH, Chu P, Hart NK, Lane A. *Toxicol Appl Pharm.* 1982; 63:351–362.
22. Raisfeld IH. *Toxicol Appl Pharm.* 1980; 56:326–336.
23. Tanaka W, Takita T. *Heterocycles.* 1979; 13:469–476.
24. Oka S. *Recent Results Cancer Res.* 1980; 74:163–171. [PubMed: 6160600]
25. Raisfeld IH, Kundahl ER, Sawey MJ, Chovan JP, Depasquale J. *Clin Res.* 1982; 30:A437.
26. Raisfeld IH. *Clin Pharmacol Ther.* 1981; 29:274.
27. Raisfeld IH. *Clin Res.* 1980; 28:A530.
28. Raisfeld IH. *Clin Res.* 1979; 27:A445.
29. Takahashi K, Aoyagi HS, Koyu A, Kuramochi H, Yoshioka O, Matsuda A, Fujii A, Umezawa H. *J Antibiot.* 1979; 32:36–42. [PubMed: 83987]
30. Ito K, Handa J, Irie Y, Ezura H, Kumagai M, Irie Y, Suzuki A, Hagiwara T, Yamane H, Miyamoto K, Yamashita T, Tsubosaki M, Matsuda AN, Konoha N. *J Antibiot.* 1979; 32:387–450.
31. Ishizuka M, Takayama H, Takeuchi T, Umezawa H. *J Antibiot.* 1967; 10:15–24.
32. Ishizuka M, Takayama H, Takeuchi T, Umezawa H. *J Antibiot.* 1966; 19:260–271. [PubMed: 4961685]
33. Gobbi PG, Federico M. *CRC Cr Rev Oncol-Hem.* 2012; 82:18–24.
34. Lehmann TE, Ming L-J, Rosen ME, Que L Jr. *Biochemistry.* 1997; 36:2807–2816. [PubMed: 9062108]
35. Lehmann TE, Serrano ML, Que L Jr. *Biochemistry.* 2000; 39:3886–3898. [PubMed: 10747776]

36. Lehmann TE. *J Biol Inorg Chem*. 2002; 7:305–312. [PubMed: 11935354]
37. Lehmann TE, Li Y. *J Antibiot*. 2012; 65:25–33. [PubMed: 22068157]
38. Lehmann TE, Li Y. *J Biol Inorg Chem*. 2012; 17:761–771. [PubMed: 22481631]
39. Li Y, Lehmann TE. *J Inorg Biochem*. 2012; 111:50–58. [PubMed: 22484500]
40. Feigon J, Denny WA, Leupin W, Kearns R. *J Med Chem*. 1984; 27:450–465. [PubMed: 6708048]
41. Batist G, Andrews JL Jr. *JAMA-J Am Med Assoc*. 1981; 246:1449–1453.
42. Cooper JAD Jr, White DA, Matthay RA. *Am Rev Respir Dis*. 1986; 133:321–340. [PubMed: 3511808]
43. Ginsberg SJ, Comis RL. *Semin Oncol*. 1982; 9:34–31. [PubMed: 6176027]
44. Muggia FM, Louie AC, Sikic BI. *Cancer Treat Rev*. 1983; 10:221–243. [PubMed: 6198083]

Fig. 1.
Structures of BLM-A₂, -A₅, -B₂, and PEP.

Fig. 2. Imino region of the 1D ^1H -NMR spectra in H_2O collected at 5°C for free OL and OL bound to Zn(II)BLM-A_2 , $-\text{A}_5$, $-\text{B}_2$, and Zn(II)PEP . Zn(II)BLM:DNA molar ratio 1:1.

Fig. 3. Aromatic region of the ^1H -NMR spectra collected at 25 °C in D_2O of the free MBLMs and MBLMs bound to OL. Zn(II)BLM:DNA molar ratio 1:1.

Fig. 4. OL signals in H₂O. a Bases, b Imino, and c Methyl regions of the NOESY spectra collected at 5 C ° of free OL displaying ¹H-¹H connectivities detected in D₂O (black) and H₂O (red). The different cross-signals are labeled and their assignments are compiled in Supplementary Table S2.

Fig. 5. Overlays of the NOESY spectra collected at 5 °C in H₂O for free OL (black) and OL bound to MBLMs (red). The missing NOE signals in the spectra of bound OL are indicated by arrows. These NOEs have been compiled in Supplementary Table S3

Fig. 6. Imino region of the NOESY spectra collected at 5 °C in H₂O for OL bound to MBLMs. Signal labeling is the same as in Fig. 4B. The missing NOEs are compiled in Supplementary Table S3

Fig. 7. Schematic representation of free OL displaying the ^1H - ^1H NOE connectivities detected at 5 °C in H₂O. The NOEs presented here as lines are compiled in Supplementary Table S2 and Fig. 4

Fig. 8. Schematic representation of OL bound to MBLMs displaying the ^1H - ^1H NOE connectivities detected at 5 °C in H_2O . The NOEs presented here as lines are compiled in Supplementary Table S2 and Fig. 4

Fig. 9.
a Base region of the NOESY spectra collected at 25 °C in D₂O for free (black) and bound (red) OL. b Schematic representation of OL bound to BLMs indicating the DNA bases exhibiting significant (δ more than or equal to 0.04 ppm) shifts upon MBLM complexation in red.

Fig. 10.

Overlays of the NOESY spectra collected at 25 °C in D₂O for free (black) and bound (red) OL. These spectra show the changes in the NOESY spectrum of free OL regarding the inter-base (b-s) NOEs upon MBLM complexation. The missing NOEs in MBLM-bound OL are compiled in Supplementary Table S5

Fig. 11.

Schematic representation of free OL displaying the inter-base ^1H - ^1H NOE connectivities detected at 25 °C in D_2O . Base-base (b-b, green) and base-sugar (b-s, blue). The NOEs presented here as lines are compiled in Supplementary Table S6. Dashed lines are used to avoid confusion in busy sectors.

Fig. 12. Schematic representation of OL bound to MBLMs displaying the inter-base ^1H - ^1H NOE connectivities detected at 25 °C in D_2O . Base-base (b-b, green), base sugar (b-s, blue), and NOEs only found upon MBLM binding (red). The NOEs presented here as lines are compiled in Supplementary Table S6.

Table 1
Differences in chemical shift between the NH₂⁺ and NH protons in free OL and OL bound to Zn(II)BLMs

Assignment	Chemical shifts (F2(ppm))						
	OL-A ₂	δ (A ₂) ^a	OL-A ₅	δ (A ₅) ^a	OL-B ₂	δ (B ₂) ^a	δ (PEP) ^a
C4NH _{2b}	8.00	0.03	7.99	0.03	8.01	0.02	8.01
C4NH _{2a}	6.27	-0.03	6.27	-0.04	6.26	-0.03	6.25
C5NH _{2b}	8.24	0.10	8.22	0.12	8.23	0.11	8.29
C5NH _{2a}	6.78	0.00	6.77	0.01	6.78	0.00	6.77
C12NH _{2b}	7.91	0.02	7.91	0.03	7.92	0.02	7.92
C12NH _{2a}	6.22	0.00	6.23	-0.02	6.22	0.00	6.21
C13NH _{2b}	8.17	0.09	8.15	0.11	8.16	0.10	8.21
C13NH _{2a}	6.84	0.03	6.82	0.04	6.83	0.03	6.84
G2NH	12.78	-0.02	12.78	-0.02	12.80	-0.03	12.76
G3NH	12.81	-0.02	12.80	0.00	12.84	-0.05	12.79
G10NH	12.99	-0.11	12.92	-0.03	12.97	-0.09	12.92
G11NH	12.84	-0.06	12.83	-0.04	12.82	-0.04	12.80

^a Calculated as (chemical shift in free OL)-(chemical shift in BLM-bound OL)

Table 2

Chemical shifts of the base protons in OL free and bound to Zn(II)BLMs for samples in D₂O at 25 °C

Base ¹ H	Chemical shifts (ppm)									
	OL	OL-A ₂	δ(A ₂) ^a	OL-A ₅	δ(A ₃) ^a	OL-B ₂	δ(B ₂) ^a	OL-PEP	δ(PEP) ^a	
A1H8	7.93	7.93	0.00	7.92	0.01	7.93	0.01	7.93	0.00	
G10H8	7.87	7.98	-0.11	8.00	-0.13	8.03	-0.16	8.01	-0.14	
G2H8	7.79	7.83	-0.04	7.84	-0.04	7.85	-0.06	7.84	-0.05	
G3H8	7.73	7.73	0.00	7.73	0.00	7.74	-0.01	7.74	-0.01	
G11H8	7.73	7.76	-0.03	7.76	-0.03	7.77	-0.04	7.77	-0.04	
C13H6	7.62	7.62	-0.01	7.60	0.02	7.62	0.00	7.61	0.01	
T14H6	7.56	7.55	0.01	7.54	0.02	7.55	0.02	7.55	0.02	
T6H6	7.53	7.54	-0.02	7.56	-0.03	7.57	-0.05	7.57	-0.04	
T8H6	7.55	7.56	-0.01	7.49	0.07	7.52	0.03	7.53	0.03	
C5H6	7.49	7.49	0.00	7.48	0.01	7.49	0.00	7.49	0.00	
T7H6	7.46	7.44	0.01	7.41	0.05	7.43	0.02	7.44	0.02	
C12H6	7.38	7.39	-0.01	7.38	0.00	7.39	0.00	7.38	0.00	
C4H6	7.32	7.34	-0.02	7.35	-0.03	7.35	-0.03	7.34	-0.02	
T9H6	7.29	7.33	-0.04	7.35	-0.06	7.35	-0.06	7.34	-0.05	

^a Calculated as (chemical shift in free OL)-(chemical shift in BLM-bound OL)