Nucleic Acid Recognition by Metal Complexes of Bleomycin

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Contents

I. Introduction

The bleomycins are a group of glycopeptide-derived natural products isolated from *Streptomyces verticillus*[1,2] that are clinically employed in the treatment of several neoplastic diseases[3] including squamous cell carcinomas,[4] non-Hodgkin's lymphomas,[5] testicular carcinomas,[6] and ovarian cancer.[7] While the bleomycin group contains over 200 closely related compounds,[8,9] the administered form of bleomycin, Blenoxane, consists mainly of bleomycins A_2 and B_2 (Figure 1). Blenoxane is administered either alone or in combination chemotherapy regimens[10−12] with, for example, *cis*-diamminedichloroplatinum(II), cisplatin, also reviewed in this issue.[13,14] The generally accepted basis for the clinical efficacy of the bleomycins is believed to derive from their ability to mediate DNA strand scission,[15] a process that produces both single- and double-strand[16,17] breaks with the latter thought to contribute significantly to the observed

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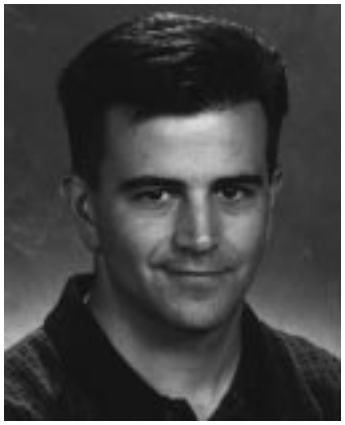

Craig Claussen was born in 1970 in Lafayette, IN. He received his B.S. degree in Chemistry from Purdue University—West Lafayette in 1991. Upon graduation, he was employed by Boehringer Mannheim and The Liposome Company before sensibly beginning his doctoral studies in 1998. He is currently a Ph.D. student at Indiana University Purdue University—Indianapolis performing research under the direction of Professor Eric Long. His current research focuses on the combinatorial synthesis of metallopeptides as nucleic acid recognition agents.

Eric Long was born in Reading, PA, in 1962. He received his B. S. degree in Biochemistry from Albright College in 1984 and subsequently completed his Ph.D. with S. M. Hecht at the University of Virginia in 1988. His Ph.D. research involved the study of various metallobleomycins and the interaction of Fe·bleomycin with structurally modified oligonucleotides. Upon completion of his Ph.D. degree, he worked as a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research under the supervision of J. K. Barton at Columbia University and the California Institute of Technology. Since 1991, he has been a faculty member within the Purdue School of Science at Indiana University Purdue University—Indianapolis (IUPUI) where he is pursuing studies of nucleic acid recognition by Ni(II)·metallopeptides, other structured peptides, and antitumor agents. He is currently an Associate Professor of Chemistry.

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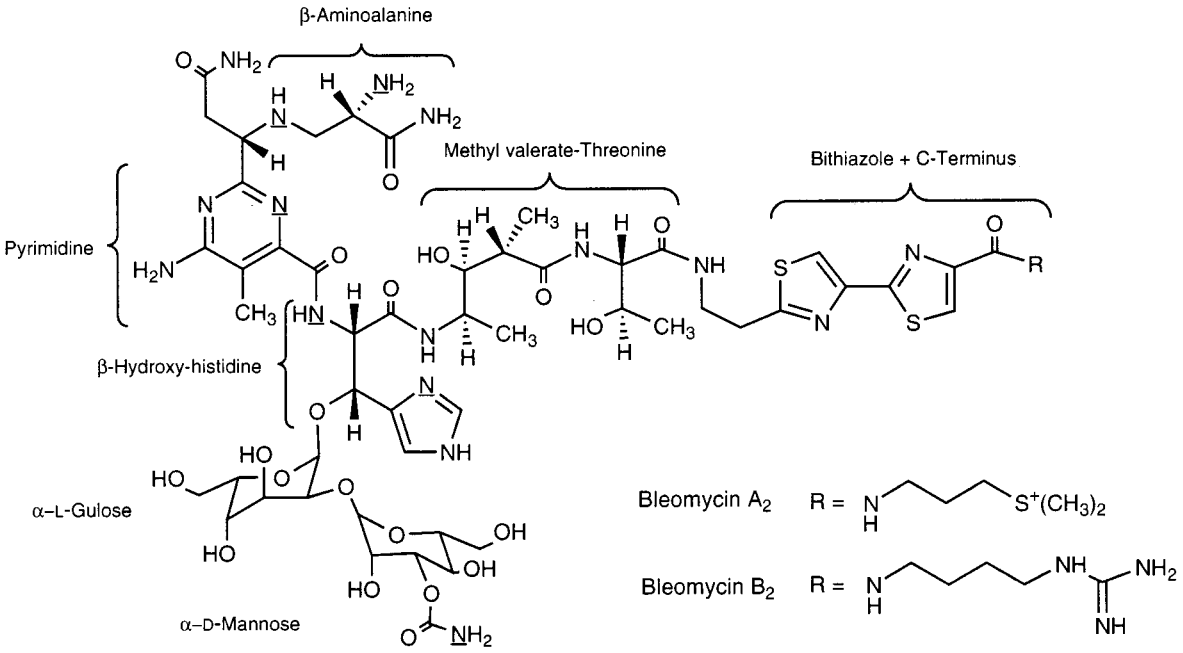


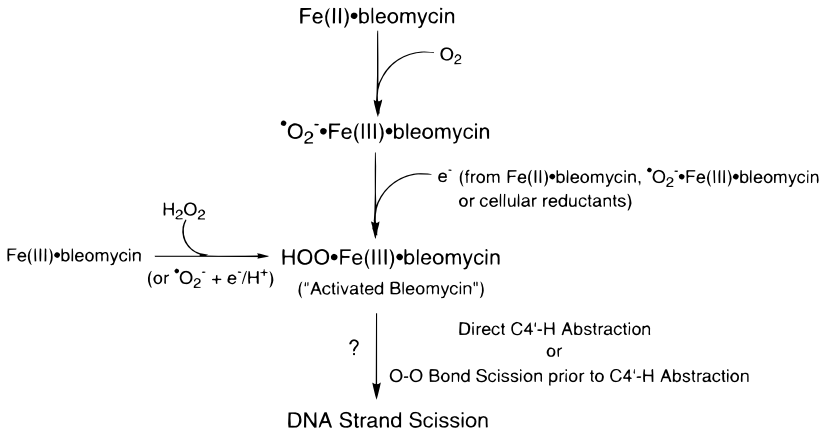
Figure 1. Structures of bleomycin A_2 and B_2 illustrating the various functional domains of the molecule. Nitrogens involved in metal ion binding are underlined.

cytotoxicity of the drug.[18,19] The process of DNA cleavage in vitro was demonstrated early on to be dependent upon metal ions and oxygen,[15,20−22] which led to the discovery of the activity[23,24] of Fe(II)·bleomycin + O_2, resulting in extensive mechanistic studies of this metallobleomycin[25−29] and derivatives thereof.[30] DNA cleavage by Fe(II)·bleomycin was also found to be a selective process; strand scission is initiated through the exclusive abstraction of the C4′−H[31,32] mainly at pyrimidine nucleotides of preferred sites containing 5′-GC and 5′-GT sequences.[33,34] Along with DNA cleavage, the bleomycins also mediate lipid peroxidation[35,36] and membrane damage[36] (which may contribute to the pulmonary toxicity of this agent[37]), the oxygenation of low molecular weight substrates,[25,26] and the selective cleavage of RNA.[38]

As determined through the first total syntheses of bleomycin,[39,40] the overall structure of this agent can be thought of as containing four distinct regions (Figure 1) consisting of (1) an N-terminal domain, which is responsible for metal binding,[26,41] oxygen activation,[25−29] and site-selective DNA cleavage,[42,43] connected via (2) a methylvalerate−threonine linker peptide to (3) a C-terminal domain, containing a bithiazole moiety which provides the majority of the DNA binding affinity,[27,44] and (4) a disaccharide moiety consisting of gulose and mannose sugars connected to the metal binding domain; the disaccharide may influence metal ion binding,[41] cell surface recognition,[38] and the selective accumulation of bleomycin in some cells[8] and possibly provides a pocket for oxygen activation.[45] Bleomycin can thus be thought of as a naturally occurring metallopeptide in its active form; while Fe has been the most extensively studied metal ion cofactor with regard to the cleavage of nucleic acids, other transition metals[46] including Cu, Co, Mn, Ni, Ru, V, and Zn bind to bleomycin and, in some instances, promote DNA strand scission. Notably, the bleomycins are isolated as Cu(II) complexes, although clinically administered in the apo-form. Thus, while evidence pointing toward Fe as the relevant cofactor exists,[47] the identity of the metal ion responsible for DNA degradation in vivo remains a subject of debate. As with other DNA binding and cleaving metal complexes,[48] the bleomycin ligand appears to employ the redox capabilities of a metal ion to promote nucleic acid scission and also to impart a defined structure to the metallodrug to effect nucleic acid recognition.

Given their multifaceted nature, the bleomycins have provided the disciplines of chemistry, biochemistry, biology, and medicine with an intriguing problem for more than 30 years. Indeed, within the fields of chemistry and biochemistry alone, bleomycin has presented a synthetic challenge,[30,39,40,49] a unique example of non-heme Fe oxygen activation,[50−55] a detailed glimpse at a pathway of oxidative DNA degradation,[25−28] and a prototypic[26] DNA cleavage agent which has helped foster the development of metal-based nucleic acid cleavage reagents.[56] While many aspects of the activity of the bleomycins have been examined in detail and are reasonably well understood, fundamental questions concerning their mechanism of action still remain unanswered. For example, the relevance of particular metal ions in vivo, the final steps in oxygen activation and utilization, and the contribution of alternative targets[38] to bleomycin cytotoxicity all need to be addressed for a full understanding of these agents. In addition, detailed knowledge of the exact means by which a metallobleomycin selectively recognizes its DNA target and orients an activated metal center to produce single- and double-strand scission via C4′−H oxidation is only now coming to light.[18,19] Information pertaining to this important aspect of the activity of the bleomycins is vital to the further development of analogues with increased clinical efficacy and our understanding, in general, of the molecular recognition of nucleic acids.

Scheme 1. Pathways of Fe(II)·bleomycin Activation



Given the very recent appearance of a review in this journal,[29] which emphasized our current knowledge of the mechanisms of bleomycin activation and DNA degradation, the review contained herein will endeavor to complement the above by focusing on our current awareness of the means by which metallobleomycin recognizes DNA resulting in site-selective strand scission. Thus, following this Introduction will be an overview of metallobleomycin activation and DNA cleavage highlighting the key points recently reviewed followed by an examination of our current understanding of metallobleomycin structure and DNA recognition.

II. Metallobleomycin Activation and DNA Cleavage–An Overview

The mechanisms of bleomycin activation and DNA strand scission have been extensively investigated in the presence of Fe(II) and O_2. While Fe(II)·bleomycin was the first of the metallobleomycins demonstrated to effect DNA strand scission, several other metallobleomycins including complexes of Cu(I), Mn(II), VO(IV), Co(III), Ru(II), and Ni(III) also mediate DNA cleavage through various forms of activation, albeit less well characterized.[46] Thus, while Fe·bleomycin is assumed to be the therapeutically active form of metallobleomycin, the discovery of additional metallobleomycins active toward DNA strand scission raises the possibility of their physiological relevance. In particular, given that the bleomycins are isolated as Cu(II) complexes and the higher binding affinity of this metal[57] for the bleomycin ligand in comparison to Fe, it is possible that Cu has at least a transient role in drug transport,[58] activation, and/or DNA cleavage.[59]

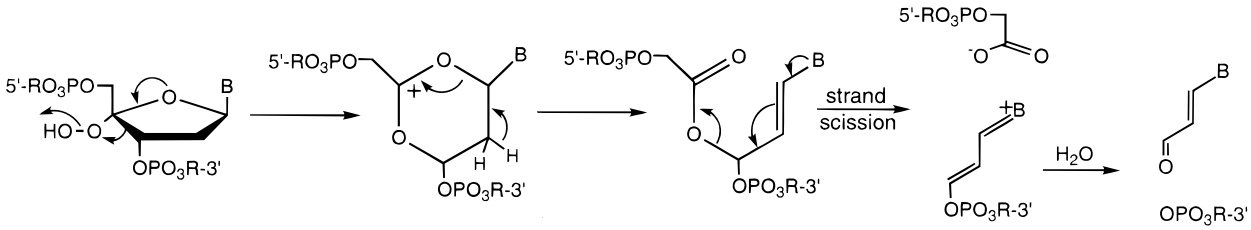
A. Fe·bleomycin

1. Oxygen Activation

In the presence of an Fe(II) cofactor, bleomycin forms an Fe(II)·bleomycin complex which reacts with O_2 in vitro[60] to create a diamagnetic species, best characterized as a ferric superoxide complex of bleomycin, $^{\bullet}O_2^{-}$—Fe(III)·bleomycin,[61] (Scheme 1). Upon formation of this initial ternary complex, two ferric superoxide equivalents of bleomycin possibly undergo disproportionation[62] to create one molecule of O_2-bound Fe(III)·bleomycin (which releases O_2 to create inactive Fe(III)·bleomycin) and one equivalent of a species commonly termed "activated bleomycin", the last detectable yet possibly penultimate species formed in the reaction of Fe(II)·bleomycin + O_2;[63] this reaction can be inhibited in the presence of high nucleotide:bleomycin ratios by presumably sequestering the ferric superoxide complex of bleomycin through DNA binding[64,65] and preventing its disproportionation. Alternatively, $^{\bullet}O_2^{-}$—Fe(III)·bleomycin may oxidize an additional equivalent of Fe(II)·bleomycin to produce "activated bleomycin" + Fe(III)·bleomycin. In addition to the above means of generating "activated bleomycin", preformed Fe(III)·bleomycin can react directly with H_2O_2[63] or $^{\bullet}O_2^{-}/e^{-}$[29,66] to create a species spectroscopically identical to that of "activated bleomycin" produced by Fe(II) + O_2.[63] Given that under cellular conditions the ratio of nucleotide to bleomycin dictates that virtually all drug is DNA-bound ($K_a \approx 10^5\ M^{-1}$),[67,68] activation of oxygenated Fe·bleomycin ($^{\bullet}O_2^{-}$—Fe(III)·bleomycin) can possibly occur[69] via microsomal[70] superoxide/NADPH or NADPH cytochrome P-450 reductase[71] and NADPH. While the exact nature of the activated bleomycin formed in the above reactions has been the subject of intense study,[61,63,72] which led to the proposal of three possible chemical species that vary with respect to their Fe—oxygen structure and configuration,[63] its structure has only recently come to light through the use of electrospray mass spectroscopy[73] and kinetic isotope studies.[74] These studies determined that "activated bleomycin" is a ferric peroxide complex of bleomycin [HOO—Fe(III)·bleomycin] with dioxygen bound end-on[75] to the Fe center including an apical hydrogen;[73] notably, spectroscopically identical yet chemically different species can also be attained in model compounds, i.e., Fe·PMAH,[76,77] of the metal binding domain of bleomycin.

As mentioned above, "activated bleomycin" is the last characterized intermediate in the process of Fe(II)·bleomycin + O_2 activation. While definitive proof remains to be obtained, it has been suggested that "activated bleomycin" might spontaneously decay[27–29,63] via O—O bond heterolysis or homolysis to produce a monooxygenated Fe·bleomycin, perhaps an O=Fe(V)·bleomycin or O=Fe(IV)·bleomycin, respectively, which

Scheme 2. Bleomycin-Induced Direct DNA Strand Scission via a C4′-Hydroperoxide Modified Nucleotide Lesion



may be the ultimate oxidant responsible for DNA damage.[29] It should be noted, however, that (1) no mass suggestive of an O−Fe•bleomycin was observed in the studies that directly examined HOO−Fe(III)•bleomycin[73] and (2) whether the process of O−O bond scission occurs before or after DNA attack is unclear. Of relevance to the above, the process of O−O bond scission within Fe(III)•bleomycin activated with the fatty acid hydroperoxide 10-hydroxy-8,12-octadecadienoic acid has been the focus of studies to determine whether O−O bond heterolysis or homolysis occurs;[78−80] upon reaction with Fe(III)•bleomycin, the products of this fatty acid hydroperoxide expected from O−O bond homolysis are released; however, the Fe•bleomycin-centered species generated did not cleave DNA efficiently.[79] These observations led to the suggestion[27,28,79] that an O=Fe(IV)•bleomycin was indeed generated in this reaction, as evidenced by its ability to modify low molecular weight substrates,[79] but this species is not competent to initiate DNA strand scission, which may require O=Fe(V)•bleomycin. In addition to the above studies with Fe•bleomycin, a recent model study[81] has demonstrated that mononuclear non-heme Fe(III)−hydroperoxides possess a weakened O−O bond that may facilitate this scission.

Formation of the ultimate Fe•bleomycin-derived DNA oxidant, in combination with the DNA binding selectivity dictated by the metallodrug structure, orients this active oxidant (which contains two oxidizing equivalents beyond the level of Fe(III)•bleomycin[72,79]) to abstract an electron exclusively from the C4′−H of a target deoxynucleotide.[31,32] This initial, rate-limiting[82−84] abstraction event produces a C4′ radical[85−87] that can partition between two pathways:[16,26] (1) the C4′ radical can recombine with an additional O_2 to produce a C4′−OO• lesion[26,88,89] that becomes reduced to form a C4′−OOH modified nucleotide[88,90] or (2) the C4′ radical can be further oxidized to produce a C4′ cation;[91] the C4′ cation ultimately adds water to produce a C4′-hydroxylated nucleotide lesion.[85]

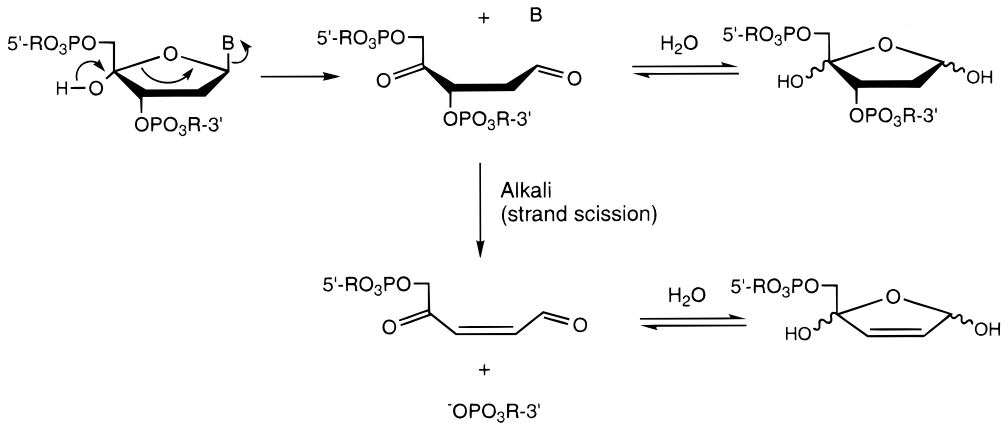
It has been speculated[29] that the above processes may be influenced by a proximally bound Fe•bleomycin; upon initial C4′−H electron abstraction, with the possible concomitant reduction of O=Fe(V)•bleomycin to O=Fe(IV)•bleomycin, the C4′−OO• lesion may serve to reoxidize the proximally bound O=Fe(IV)•bleomycin to O=Fe(V)•bleomycin, thus assisting in the formation of the C4′-OOH lesion. Alternatively, the O=Fe(IV)•bleomycin formed upon initial C4′−H electron abstraction could further oxidize the C4′ radical prior to O_2 recombination to generate a C4′ cation and Fe(III)•bleomycin. Importantly, the above partitioning and possible regeneration of an active O=Fe(V)•bleomycin DNA oxidant has been postulated as a basis for double-strand cleavage events;[29] the reformed O=Fe(V)•bleomycin may relocate or reorient to another nucleotide on the complementary DNA strand in close proximity to the initial DNA lesion to produce a second DNA C4′-oxidation event.[18]

2. DNA Cleavage Mechanisms

DNA oxidation via C4′−H electron abstraction by the ultimate Fe•bleomycin-derived species competent in this reaction results in the formation of two types of DNA lesions, containing either C4′-OOH or C4′-OH modified nucleotides. The products resulting from these lesions and the mechanisms of their formation have been examined and reviewed in detail.[25−29] As summarized in Scheme 2, the C4′-OOH lesion is believed to undergo a Creigee-type rearrangement resulting in oxygen insertion between the C4′ and C3′ carbons of the target nucleotide and the formation of a C4′ cation. This rearrangement is likely facilitated by the proximity of an Fe•bleomycin "catalyst" given the stability of synthetic C4′-hydroperoxy nucleotides.[90] The intermediate formed in the above reaction promotes the specific elimination of the 2′-*pro-R*-proton and C1′−O bond rupture.[82,92,93] Subsequently, DNA strand scission occurs with the release of a new DNA terminus bearing a 3′-phosphoroglycolate moiety.[94] The remaining nucleotide fragment[29] connected to the new 5′-terminus formed at the site of strand scission then hydrolyzes through the addition of solvent to the 3′ carbon to release a nucleobase propenal[94] and a new 5′-phosphorylated DNA terminus.[95] The mechanistic pathway described above and depicted in Scheme 2 follows the observation that DNA strand scission and 2′-*pro-R*-proton release occur at comparable rates and that both of these processes occur more rapidly than nucleobase propenal release and the formation of new 5′-phosphorylated DNA termini.[96−98]

In contrast to the C4′-OOH lesion which results in direct DNA strand scission, C4′-hydroxylation[82,85] results only in the release of free nucleobase[99] and the creation of an alkaline-labile lesion[16] that does not result in strand scission in the absence of further chemical workup. As shown in Scheme 3, C4′-hydroxylation leads to elimination of the N-glycosidic bond and release of a free nucleobase to create a ketoaldehydic abasic lesion.[100−102] This abasic lesion is then prone to labilization of the C2′−H in the presence of alkali, resulting in strand scission via the elimination of a new DNA termini bearing a 5′-phosphate.[100−102] The intermediates in this reaction

Scheme 3. Bleomycin-Induced Alkaline-Labile Site Formation via a C4′-Hydroxylated Nucleotide Lesion and DNA Cleavage upon Treatment with Alkali



5'-G-**Py**-Py-Pu-3'	5'-G-**Py**-Pu-Py-3'	5'-G-**Py**-Pu-Pu-3'
3'-C-Pu-Pu-Py-5'	3'-C-Pu-Py-Pu-5'	3'-C-Pu-Py-Py-5'

Figure 2. Sequence selectivity of bleomycin-induced double-strand DNA scission. Bold and underlined pyrimidine (Py) residues depict primary and secondary sites of DNA modification, respectively. (Adapted with permission from ref 18. Copyright 1996 American Chemical Society.)

have been examined through various means including a determination of the protons released upon treatment with alkali,[82] direct product analysis and comparison to synthetic models,[101,102] chemical trapping,[102] and $NaBH_4$ reduction coupled to direct MS analysis.[103]

3. Double-Strand DNA Cleavage

The DNA modification pathways described above can occur at isolated sites, predominantly within 5′-GC and 5′-GT sequences, resulting in single-strand events of DNA cleavage. In addition, Fe·bleomycin is also capable of inducing strand scission via this chemistry on both complementary strands of a DNA target (10−20% of the lesions formed) to produce double-strand cleavage.[16−19,104] Double-strand cleavage sites (Figure 2) lead to blunt ended DNA termini, when the two acts of cleavage involve complementary nucleotides, or termini with single nucleotide 5′ extensions, when the two acts of DNA modification occur at nucleotides on complementary strands that are 5′-staggered by one base pair.[105−107] Importantly, double-stranded DNA cleavage appears to correlate well to the bleomycin-induced inhibition of cell proliferation, most likely since it is repaired more slowly and less efficiently relative to single-strand cleavage events.[19] Thus, understanding the mechanism(s) of double-strand cleavage and the features of metallobleomycin that lead to it could assist in the development of bleomycin analogues with increased clinical efficacy.

Double-strand DNA cleavage by Fe·bleomycin does not appear to result from the simple accumulation of single-strand cleavage events, rather it has been inferred based on a probable distribution analysis[16,108] to occur through a single metallobleomycin binding event which results in the modification of two different nucleotides in close complementary proximity.[29,106,108] Further, the simple accumulation of single-strand cleavage events within close proximity would be highly unlikely under in vivo conditions of extremely high nucleotide:metallobleomycin ratios.[109] Additional support for the involvement of a single metallobleomycin binding event was provided by examination of the site-selectivity of double-strand DNA cleavage (Figure 2);[105−108] generally only one of the two nucleotide sites involved in a double-strand cleavage site contain the preferred 5′-G−Py sequence with the second act of cleavage determined by whether there is a purine or pyrimidine residue 3′ to the Py in the preferred site of cleavage.[105,106] This observation led to the proposal of the presence of both primary and secondary binding sites at a location of double-strand cleavage with the primary site believed to be the first location of Fe·bleomycin association and C4′ modification.[105,106]

Thus, depending upon the DNA sequence targeted, it appears that metallobleomycin may be capable of associating with a preferred (primary) site of cleavage leading to an initial act of C4′ modification followed by either (1) total drug−DNA dissociation (leading to single-strand cleavage) or (2) a repositioning[18,107] of the same metallobleomycin by 15−18 Å to an additional nucleotide site on the complementary DNA strand leading to a second event of C4′ modification. This model[18,105] for double-strand DNA cleavage necessitates that the original Fe·bleomycin be reactivated[18,29] and competent for C4′ abstraction. Thus, as described in the previous section, C4′−H abstraction by O=Fe(V)·bleomycin leading to the formation of a peroxyl radical at the primary site followed by reoxidation of O=Fe(IV)·bleomycin by the peroxyl radical may regenerate an active O=Fe(V)·bleomycin which can reposition and lead to a second act of C4′ modification.[29] This interesting proposal is supported by the observation that the primary sites of double-strand cleavage only occur through C4′-OOH intermediates, the formation of which may serve to reactivate Fe·bleomycin, and that double-strand cleavage sites never contain a pair of alkaline labile lesions.[110]

Previous studies have shown that double-strand cleavage by bleomycin occurs as a function of both the sequence[105−108] of the targeted DNA strand and the structure of the nicked DNA intermediate[111,112] formed at the primary site of Fe·bleomycin interac-

tion. In addition, the structure of metallobleomycin itself profoundly influences the frequency of double-strand cleavage; studies have shown the importance of the peptide linker[113−120] that connects the metal binding domain with the bithiazole + C-terminus, leading to the suggestion[18] that the linker acts as a flexible tether that allows a single bithiazole anchoring event to lead to both primary and secondary positionings of the metal binding domain at a site of double-strand cleavage; ongoing studies will no doubt determine the relevance of this current model to some or all sites of double-strand DNA cleavage by metallobleomycin. The possibility that Fe·bleomycin can bind DNA, induce one DNA modification event, become reactivated, and without total dissociation, reposition to another nucleotide and induce C4′-abstraction is very intriguing and underscores the unique abilities of this anticancer agent. While many details of the process by which Fe·bleomycin generates double-strand cleavage events are not understood fully, further insight as to the basis for double-strand cleavage by bleomycin have been provided by the examination of DNA-bound metallobleomycins amenable to NMR analyses.[18,113,114]

B. Other Metallobleomycins

While metallobleomycins in addition to Fe(II)·bleomycin are capable of effecting DNA cleavage, in general, much less is known about their mechanisms of activation and DNA strand scission.[26,46] In the case of Cu·bleomycin, the structure of which has been reviewed,[26,41] early observations indicated that Cu inhibited the action of bleomycin.[24] However, it was later demonstrated that Cu(I)·bleomycin[121] could be aerobically oxidized to generate oxygen radicals,[122] leading to the finding that Cu(II) + bleomycin in the presence of dithiothreitol was capable of cleaving DNA.[123] While concerns have been raised that the activity of Cu(I)·bleomycin may be due to contaminating Fe,[124] conditions have been delineated that clearly demonstrate the activity of Cu(I)·bleomycin when reductively activated.[59] Notably, DNA damage by Cu(I)·bleomycin, while found to mediate cleavage mainly at 5′-GC and 5′-GT sequences and to produce both free nucleobase and nucleobase propenals, differed both qualitatively and quantitatively from that exhibited by Fe(II)·bleomycin.[59] Also demonstrated was the fact that Cu + Fe could increase DNA cleavage beyond that exhibited by either metal ion alone.[59] This latter finding suggests that even if Cu is not involved directly in producing DNA damage in vivo, this metal may assist in generating an active Fe·bleomycin under cellular conditions.

While likely of less direct therapeutic relevance, DNA cleavage by several other metallobleomycins has also been investigated. Among these, the Co complex of bleomycin is important as a model[18,19] of Fe(II)·bleomycin activation and structure due to its ability to bind bleomycin and O_2 to form a ternary complex[125−130] that appears to closely resemble oxygenated Fe(II)·bleomycin; aerobic oxidation of Co(II)·bleomycin in situ results in the formation of two Co(III)·bleomycin species: a peroxide complex, HOO−Co(III)·bleomycin, and Co(III)·bleomycin alone.[131,132] These metallobleomycins, designated Co(III)·bleomycin "green" and "brown", respectively,[128] are both capable of mediating DNA cleavage upon photoirradiation[133−137] with the former species being the more efficient of the two. Investigations of DNA cleavage by these complexes revealed the formation of alkalilabile sites, the release of free nucleobases,[137] and DNA termini containing 3′-phosphoroglycolates.[134] However, in contrast to Fe·bleomycin, nucleobase propenals, which would be expected to accompany the formation of phosphoroglycolate termini, were not detected. These and further studies indicate that HOO−Co(III)·bleomycin, upon photoirradiation, initiates DNA strand scission through C4′−H abstraction[137] resulting in site-selective damage[133] that parallels Fe·bleomycin but includes additional sites of DNA modification that may not correlate to sites of drug−DNA binding;[138] this observation and the lack of nucleobase propenal formation suggests that some fundamental differences in the reactivities of Fe·bleomycin and Co·bleomycin exist. In addition to Co(III)·bleomycin, several other metallobleomycins can be activated toward DNA strand scission through even less well examined mechanisms including Ru(II)·bleomycin + $h\nu/O_2$;[139] Mn(II)·bleomycin + O_2,[140,141] H_2O_2[142] or $h\nu$;[141] VO(IV)·bleomycin + H_2O_2;[143] and Ni(III)·bleomycin[144] through guanine nucleobase oxidation.

III. Metallobleomycin Structure and Studies of DNA Recognition

While limited to the realm of speculation for many years, knowledge of how metallobleomycin recognizes and binds to a nucleic acid target is central to our understanding of the function of this antitumor agent. Such information would provide insight into the means by which metallobleomycin selectively binds to 5′-GC and 5′-GT sites[33,34] and orients a reactive oxygen equivalent to exclusively react with the C4′−H of a target nucleotide.[31,32] Importantly, our understanding of these features of metallobleomycin activity could perhaps lead to the development of analogues with greater clinical efficacy. Toward understanding the above, it has become clear in recent years that the functions of oxygen activation/delivery and site-selectivity by metallobleomycin both are contained, in part, within the metal binding domain of this agent.[42,43] Thus, our understanding of these features of bleomycin activity are intimately linked to knowledge of the three-dimensional structure of the metal-bound drug. Toward this end, recent studies have furthered our understanding of the structures of various metallobleomycins. In particular, investigations employing metallobleomycins amenable to NMR techniques are also providing us with the wherewithal to examine DNA-bound structures at increased resolution.

A. Fe·bleomycin

The Fe complex of bleomycin is generally considered to be the metallobleomycin of therapeutic relevance. Unfortunately, the exact structure of this metallobleomycin in the absence or presence of DNA

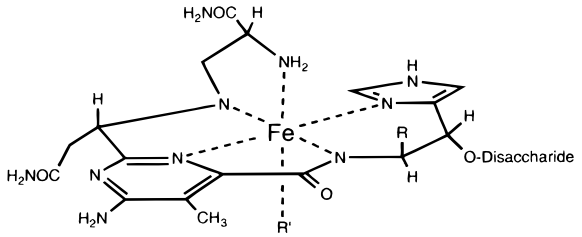


Figure 3. Proposed structure of Fe·bleomycin. R and R′ denote the connection via the methyl valerate−Thr linker peptide to the bithiazole + C-terminus and the likely location of oxygen or carbamoyl nitrogen ligation, respectively. This structure reveals one of two possible screw sense isomers that may form upon complex formation.

has remained controversial for many years.[26,41] Thus, in lieu of direct structural information via crystallography or NMR, many studies have focused on defining the interactions that occur between DNA and Fe·bleomycin through ESR, resonance Raman, and other spectroscopies, the use of modified DNA substrates, and synthetic analogues of bleomycin in an attempt to study structure−function relationships.

1. Structure

The structure of Fe·bleomycin has been a topic of considerable interest since the discovery of its DNA cleavage activity. Early spectroscopic studies, as thoroughly reviewed,[26] suggested that Fe(II)·bleomycin displays a five-coordinate, square pyramidal geometry with the secondary amine of β-aminoalanine, N1 of pyrimidine, and His imidazole equatorially ligated to the central Fe(II) atom; Fe(III)·bleomycin was thought to be analogous but with a hydroxyl ligand occupying an axial coordination site and completing an octahedral geometry. The identity of the remaining ligands in Fe(II)·bleomycin remained in dispute, with EPR studies[145] of nitrosyl ternary complexes suggesting axial ligation of the β-aminoalanyl primary amine and equatorial ligation of the deprotonated β-hydroxyimidazole amide nitrogen (Figure 3). In contrast, NMR experiments that examined the low-spin, diamagnetic CO−Fe(II)·bleomycin ternary complex, a potential model for the ligand arrangement in oxygenated Fe·bleomycin, suggested an alternative coordination environment in which the equatorial β-hydroxyimidazole amide does not ligate, rather the fourth equatorial site is occupied by the β-aminoalanyl primary amine with the carbamoyl nitrogen acting as an axial ligand.[146,147]

Since this early work, several studies have been carried out on the Fe(II) and Fe(III) complexes of bleomycin which have served to shed light on a possible consensus structure. Utilizing 2D ^{1}H and ^{13}C NMR techniques to reexamine CO−Fe(II)·bleomycin, one study[148] has proposed a structure which includes the secondary amine of β-aminoalanine, pyrimidine N1, β-hydroxyhistidine amide, and imidazole as equatorial ligands, in agreement with elements of earlier studies. In this structure, the mannose carbamoyl is bound permanently as one axial ligand with CO occupying the sixth coordination site; the primary amine of β-aminoalanine was not observed to participate in metal complexation in this study. Since the CO complex was used as a model for the ligand arrangement within oxygenated Fe·bleomycin, this structure suggested that this sixth binding site is actually the location of oxygen binding in the activated species. More recently,[149] a direct NMR study that took advantage of the paramagnetic nature of Fe(II)·bleomycin supports an alternative structure in which the equatorial ligands are the same as above,[148] but with the β-aminoalanyl primary amine occupying the fifth (axial) coordination site in lieu of the mannose carbamoyl nitrogen. Along with these direct studies of Fe·bleomycin, synthetic models of the metal binding site of bleomycin (PMAH)[76] which include only donor nitrogens representing the primary and secondary amines of β-aminoalanine, pyrimidine, β-hydroxyhistidine amide, and imidazole have been found to bind metals in a fashion similar to bleomycin; these results provide further support for the structure proposed in the paramagnetic NMR study[149] and earlier studies.[145]

In addition to the above, resonance Raman investigations[150] of the Fe(II), Fe(III), and CO−Fe(II) complexes of bleomycin implicate participation of the β-hydroxyhistidine amide and pyrimidine (along with the imidazole and secondary amine as equatorial ligands and the primary amine of aminoalanine as an apical ligand) in the coordination environment of all three forms of Fe·bleomycin. These authors also observed changes in the resonance Raman spectra of Fe(III)·bleomycin upon binding to d(CG)$_3$[151] which point toward small conformational changes in these two ligands as well as in the Fe−OH ligand environment. Importantly, these results support the notion that the metal binding domain binds to DNA in addition to the bithiazole + C-terminus[150] and also provide observations of the Fe·bleomycin complex directly bound to DNA.

Very recently, spectroscopic observations of high-spin Fe(II)·bleomycin complexes have been reported[55] which indicate that altering the primary amine of β-aminoalanine and the mannose carbamoyl substituent affect the coordination environment of the metal center, which would seem to implicate both of these moieties in some degree of metal coordination. To resolve this apparent conflict, a dynamic structure was proposed[55] in which the primary amine of β-aminoalanine is the permanent axial ligand with the secondary amine, pyrimidine, deprotonated amide, and imidazole as equatorial ligands, but the carbamoyl weakly occupies the sixth binding site; upon displacement of the carbamoyl and possible ligand rearrangement, this site is proposed to become the location of exogenous small molecule binding, i.e., dioxygen, in the activated form of Fe·bleomycin (Figure 3). Similar studies[53] have also pointed out the importance of pyrimidine ligation; this ligand provides strong back-bonding to the metal center creating an environment conducive to stabilization of the dioxygen complex of oxygenated Fe(II)·bleomyin.

Throughout the studies described above, there is certainly adequate evidence to support the contention that the carbamoyl substituent is coordinated to the metal center at some point; however, the predominant apical coordination of the primary amine is more strongly supported. The recent report de-

scribed[55] would thus appear to bring the two conflicting views concerning carbamoyl ligation into agreement in a model in which both possible ligand binding events are accommodated. Perhaps initial weak binding of the carbamoyl to the unoxygenated Fe(II) center assists bleomycin in its competition for and effective sequestration of intracellular iron prior to a ligand structural reorganization and oxygenation.

2. DNA Structural Contributions to Drug Recognition

The original view of Fe·bleomycin activity involved DNA recognition and binding by the bithiazole + C-terminus with the metal binding domain acting mainly as a "warhead" that delivered a reactive oxidizing equivalent to an adjacent nucleotide. The picture emerging currently, however, is appreciably more complex with the "traditional" DNA binding domain (bithiazole + C-terminus) acting in concert with the metal binding domain to achieve DNA site-selectivity, binding, and efficient cleavage. While high-resolution structures of Fe·bleomycin bound to DNA are not yet available, many chemical and biochemical studies have been performed in recent years to probe the basis for drug−DNA site-selectivity and binding through an understanding of the structural contributions made by the DNA target and the various domains of metallobleomycin.

As mentioned, Fe·bleomycin and other metallobleomycins exhibit a strong preference for 5′-GT and 5′-GC sequences with C4′−H modification occurring at the pyrimidine residue of these sites. As summarized for studies prior to 1987,[26] this selectivity was believed to be achieved through a direct intercalative or minor groove binding interaction between the bithiazole and key elements of the target DNA structure, most likely the exocyclic amine of G residues (Figure 4).[152] Minor groove residence by bleomycin was implied through studies using structurally modified DNA substrates which indicated that modifications in the major groove did not inhibit strand scission[153,154] while agents bound in the minor groove altered Fe·bleomycin-mediated DNA cleavage.[153,155] Indeed, exclusive abstraction of the C4′−H, located prominently in the minor groove,[87] indicates that at least some, if not all, of the metallobleomycin structure resides in this groove upon binding. It should be noted, however, that while not believed to directly block bleomycin interaction, major groove modifications such as the presence of 5-methylcytidine residues that result in localized DNA conformational changes can diminish Fe(II)·bleomycin strand scission[156] or alter the partitioning between direct strand scission products and alkaline-labile site formation.[157] Importantly, these studies indicate that natural covalent modifications of DNA with a regulatory function may alter bleomycin selectivity and chemistry in vivo from that observed in vitro.

More recently, direct examinations of the role of the exocyclic amine of guanine residues on Fe(II)·bleomycin selectivity have been performed.[158,159] In one study,[158] inosine and 2,4-diaminopurine were systematically substituted at DNA sites that contained guanine or adenine residues, thus removing or adding an exocyclic amine at these minor groove sites, respectively (Figure 4). It was shown that when the exocyclic amine of a guanine is removed via inosine substitution, Fe(II)·bleomycin exhibited less strand scission at previously strong cleavage sites involved in 5′-GT/C sequences. Meanwhile, diaminopurine residues created new sites of cleavage at pyrimidines 3′ to this substitution. These studies indicate that while not an absolute requirement for DNA cleavage, the exocyclic amine of guanine is a critical recognition element that leads to 5′-GC and 5′-GT site-selectivity.

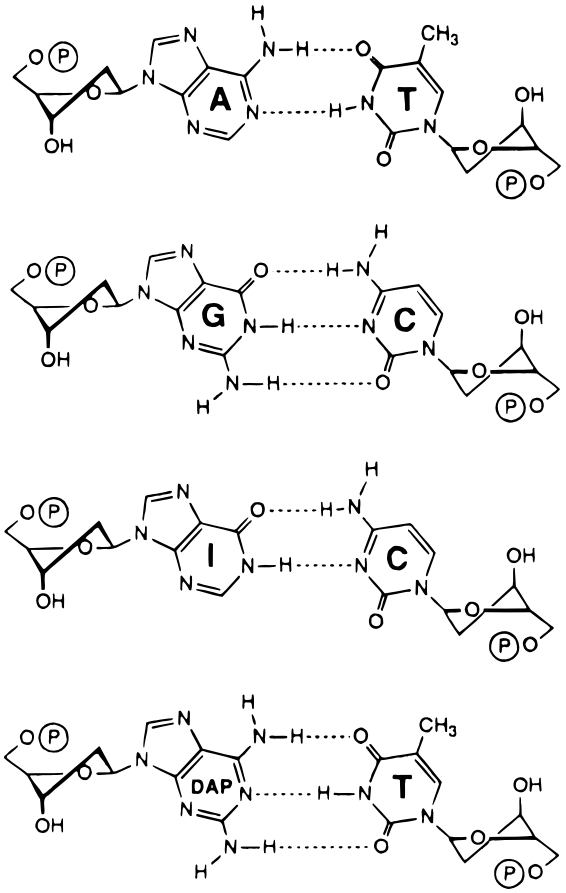


Figure 4. Structures of hydrogen-bonded purine−pyrimidine base pairs illustrating the location of the exocyclic amine of guanine. Also illustrated are the base pairs that occur upon substitution of inosine (I) for guanine and 2,6-diaminopurine (DAP) for adenine. (Reprinted with permission from ref 158. Copyright 1995 American Chemical Society.)

In an additional study,[159] oligonucleotide substrates were employed containing N^2-isobutyrylguanine moieties at specific locations within a target sequence for Fe(II)·bleomycin. Upon cleavage by Fe(II)·bleomycin, when this guanine modification occurred 5′ to a primary bleomycin cleavage site, strand scission was suppressed by greater than 10-fold in comparison to the absence of this modification. In addition, isobutyrylguanine substitutions two nucleotides 5′ to the target site or directly across from a target nucleotide also diminished bleomycin-induced DNA strand scission while modifications which occurred 3′ to a targeted nucleotide had little effect on Fe(II)·bleomycin-induced cleavage. These results suggested

that the metallobleomycin binding site encompasses two or three base pairs within the DNA minor groove including the nucleobases associated directly with the targeted nucleotide and one or two base pairs to its 5′ side. This view of the interaction of metallobleomycin is also consistent with models developed upon the examination of bleomycin cleavage of DNA oligonucleotides containing unpaired, "bulged" nucleotides.[160]

3. Bleomycin Structural Contributions to DNA Recognition

An understanding of the elements of metallobleomycin structure that interact with DNA has been addressed through studies using natural structural congeners or, increasingly, synthetic analogues of bleomycin.[30] In contrast to our earlier picture of metallobleomycin−DNA binding, several studies have shown that cleavage site-selectivity is dictated predominantly by the metal binding domain and does not lie exclusively in the bithiazole + C-terminus. In one study, bleomycin analogues with identical bithiazole + C-termini but differing in their metal binding domains exhibited altered sequence selectivities[161] and strand selectivities.[162] Conversely, bleomycin analogues containing very different C-termini but with identical metal binding domains all exhibited a 5′-GT/C selectivity as with bleomycin.[163] In a more direct study to address the role(s) of the metal binding domain on DNA selectivity, bleomycin analogues which replaced the Thr linker between the metal binding domain and the bithiazole + C-terminus with Gly_n spacers ($n = 0-4$ residues) also exhibited identical selectivities despite the altered distances between the two regions.[42,43] This study strongly suggests that the structural determinant of DNA site-selectivity resides amino-terminal to the Thr residue within the metal binding domain. An additional study[76] in support of this hypothesis has indicated that an analogue of the metal binding domain lacking the bithiazole + C-terminus (PMAH) exhibited a DNA cleavage selectivity similar to bleomycin; however, other bleomycin analogues possessing metal binding domains more similar to bleomycin failed to exhibit selective cleavage in the absence of the bithiazole + C-terminus.[30,164,165] It is possible, however, that the above discrepancy may have more to do with relative binding affinities than recognition abilities.

Along with the ability of the metal binding domain to influence cleavage site-selectivity, it has also been found that this domain can influence the overall interaction of bleomycin with DNA; Cu(II) binding was found to promote the ability of bleomycin to mediate the unwinding of supercoiled DNA through a proposed metal ion induced "allosteric" organization of the metal binding domain into a compact, well-defined three-dimensional shape that facilitates proper DNA association.[166] While these data make it clear that Cu binding to bleomycin facilitates DNA unwinding and interaction, the ligand arrangement within Cu(II)·bleomycin[26,41] may differ from that of Fe·bleomycin and thus also its mode of DNA interaction.

In addition to the above, a number of recent studies have also sought to define more specifically the functional role(s) of the metal binding domain of bleomycin through systematic synthetic alterations of particular functional groups. In one study[167] designed to investigate the significance of the His amide (a likely metal ligand upon deprotonation), synthetic analogues were prepared incorporating either (1) a tertiary *N*-methyl amide or (2) an ester in place of the amide; neither of these alterations would be expected to participate in proper metal coordination. Consistent with the belief that the His amide is required for metal ion binding, both of these analogues were found to cleave DNA significantly less efficiently and at lower double-strand:single-strand cleavage ratios in comparison to unaltered bleomycin. In addition, these analogues were also found to cleave DNA in a nonselective fashion, thus lacking the characteristic selectivity of Fe·bleomycin. In contrast, one alteration within the His residue to produce an analogue of deglycobleomycin lacking only the β-hydroxy group of His, which is not believed to participate in metal ion complexation, was found to cleave DNA in a fashion essentially identical to Fe(II)·deglycobleomycin; this observation confirmed that the differences between the activities of bleomycin and deglycobleomycin are due to the lack of the disaccharide moiety in the latter and not the introduction of a free hydroxyl group within the structure of bleomycin. The studies outlined above are thus consistent with the view of bleomycin metal binding involving the deprotonated His amide residue.

In another study,[168] focused on addressing the role of metal complexation through the imidazole-N of the β-hydroxyhistidine subunit, three analogues of deglycobleomycin were synthesized in which the imidazole ring was deleted or replaced with either an oxazole or pyrrole ring, thus altering metal ion coordination at the site of interest; these structural analogues also allowed for a comparison of N^{σ} and N^{π} coordination of the imidazole ring, since the nature of this coordination is not well established. Specifically, the oxazole-containing analogue was incapable of N^{σ}-coordination but can function as an N^{π}-donor comparable to imidazole, while in contrast the pyrrole ring can only function as an N^{σ}-donor. It was found that the DNA cleavage selectivity of the oxazole−deglycobleomycin analogue was similar to that of deglycobleomycin with slightly lower efficiency; however, the efficiency and selectivity of the pyrrole-containing analogue was significantly reduced in a manner comparable to the methylamide and ester analogues discussed in the previous paragraph.

Along with an examination of the β-hydroxy His residue, the role of the C4 amino group of the pyrimidine moiety of bleomycin in DNA recognition was studied[169] through the synthesis of deglycobleomycin analogues lacking the C4 amino group or containing a dimethylamino group in place of the C4 amino group. The dimethylamino-containing analogue was found to exhibit diminished DNA cleavage efficiency as well as a loss of sequence selectivity. Interestingly, the desamino analogue showed a more greatly diminished cleavage efficiency compared to that of the dimethylamino-containing analogue but

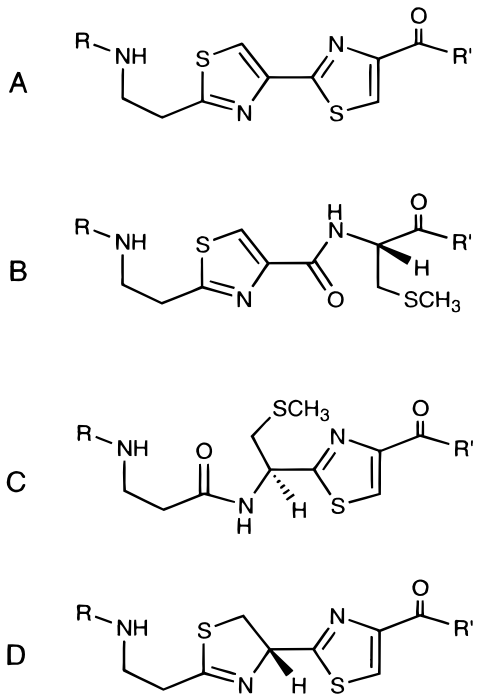


Figure 5. Structures of the unmodified bithiazole of bleomycin (A) in comparison to synthetic monothiazole derivatives (B and C) and the thiazolinylthiazole moiety of phleomycin (D). R and R′ denote the remaining portions of bleomycin, the amino terminus and carboxy terminus, respectively.

maintained some level of sequence selectivity depending upon the assay conditions employed. These results support the proposed formation of two hydrogen bonds between the pyrimidine group of bleomycin and guanine at a recognition site, as discussed in section III.B.2; most likely, the presence of the dimethylamino group sterically interferes with this interaction, leading to loss of selectivity, and lack of the amino group merely removes one of the two possible hydrogen bonds, leading to diminished selectivity. The above also is reflected in the observed cleavage at 5′-AC and 5′-AT sites which occurs at much lower efficiency, given the single H-bond possible between the pyrimidine moiety of bleomycin and an adenine located at a site of binding (see section III.B.2).

The foregoing studies make it apparent that the metal binding domain is dominant in determining the cleavage selectivity exhibited by the drug and thus by inference is likely to be the moiety that directly interacts with the exocyclic amine of G residues. This finding, in retrospect, is reasonable given the precision with which the metal binding domain must be placed to achieve exclusive C4′−H abstraction of the adjacent pyrimidine nucleotide. However, while these studies indicate that the bithiazole has a somewhat lesser role in dictating selectivity than previously thought, direct studies with modified bithiazole-containing analogues of bleomycin indicate that in order for the metal binding domain to selectively and efficiently cleave DNA, both rings of the bithiazole must remain present.[170] It was found that derivatives of bleomycin systematically lacking either of the monothiazole rings (Figure 5), while capable of activating oxygen in a fashion identical to intact Fe·bleomycin, cleaved DNA without site-selectivity; cleavage was observed to occur at all nucleotides equally. While total removal of a thiazole ring leads to loss of selectivity, it is necessary to mention that slightly altered yet intact ring systems permit bleomycin selectivity to occur as with the intact bithiazole: (1) phleomycin, a naturally occurring congener of bleomycin containing a thiazolinylthiazole ring system (Figure 5) that is thought to be unable to intercalate DNA, displays a selectivity similar to bleomycin[171] and (2) phototransformed bithiazoles within bleomycin do not alter selectivity.[172,173]

While an intact bithiazole or slight modifications thereof appear necessary to assist the metal binding domain in achieving site-selectivity, the inherent selectivity of the bithiazole moiety alone is less well understood. Studies that have attempted to examine the selectivity of the bithiazole itself by eliminating the metal binding domain and conjugating the bithiazole + C-terminus with either Fe·EDTA[174] or Co·2-(1,3-diaminopropyl)[175] substituents failed to reveal a selectivity related to that of intact Fe·bleomycin. More recently, the use of chlorinated bithiazoles, which can be photoactivated to induce DNA strand scission,[176] have been shown to induce minor groove DNA modification (via Cl· induced C1′-H and C5′-H abstraction) at sites 3′ to a high-efficiency cleavage site of Fe·deglycobleomycin.[177] When examined within Fe(II)·deglycobleomycin, photoactivated DNA cleavage by the chlorinated bithiazole moiety occurred at T_4 and A_5 of d(CGCTAGCG)$_2$ while O_2 induced activation of the metal binding domain occurred at C_3 and C_7,[177] also the preferred sites of Fe(II)·bleomycin cleavage.[178] While difficult to assess an exact binding mode, this study does, however, suggest that upon DNA association, the bithiazole lies 3′ to the 5′-GC sites cleaved by the metal binding/oxygen activation domain of intact metallobleomycin and that its mode of association permits access to minor groove protons in this DNA region. Surprisingly, in the absence of the metal binding domain of bleomycin, the chlorobithiazoles also mediated DNA damage in the same locations of this oligonucleotide substrate.[179] This observation suggests that the bithiazole does indeed display a level of inherent selectivity which, if flanking preferred sites of metal binding domain selectivity, may create high-efficiency metallobleomycin cleavage sites.

Thus, while not providing the structural determinant of cleavage selectivity, the bithiazole appears necessary to achieve adequate DNA binding and to possibly promote selectivity within the total Fe·bleomycin structure. These findings lend credence to the idea that the bithiazole may act as a cooperative partner with the metal binding domain resulting in an increase in binding affinity and to perhaps serve as a means to properly orient the metal binding domain in relation to the DNA target. While it is still not yet clear whether the bithiazole of Fe(II)·bleomycin associates with DNA through intercalation, partial intercalation, or groove binding,[18,27,28,44,114,152,166,177] a very recent study[180] in which bleomycin A_5, a naturally occurring congener containing a C-terminal spermidine moiety, was conju-

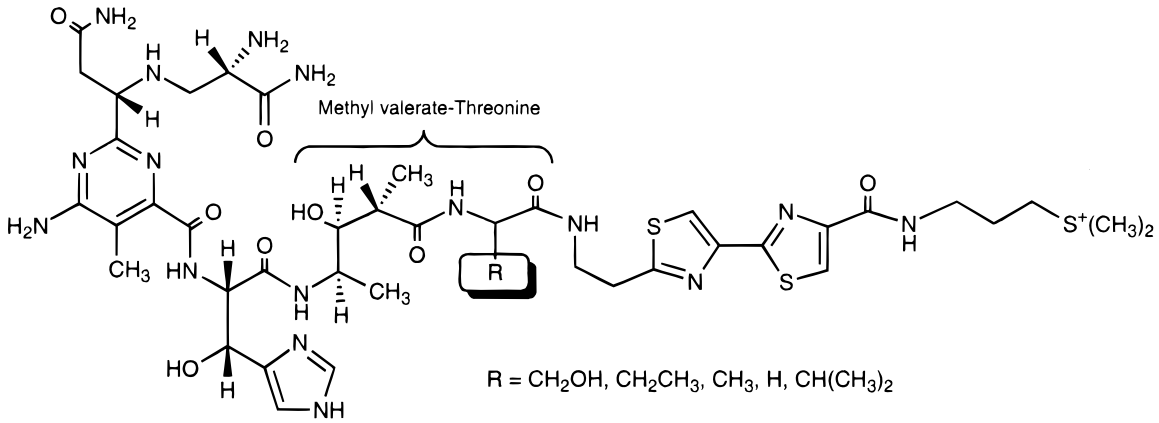


Figure 6. Illustration of the modifications made within the methylvalerate−Thr linker peptide in an examination of the function of this moiety. (Adapted with permission from ref 119. Copyright 1998 American Chemical Society.)

gated directly to a controlled pore glass (CPG) bead approximately 10^5-fold larger than bleomycin itself exhibited a DNA cleavage selectivity that was identical to nonconjugated Fe(II)·bleomycin A_5; while other modes of bithiazole−DNA interaction are not precluded, these results indicate that a "threading" type of intercalation, where the C-terminal substituent inserts through the minor groove to the major groove, is not necessary to achieve DNA cleavage and the characteristic site-selectivity of Fe(II)·bleomycin. The above study and higher resolution structural studies, to be discussed later, have led to the suggestion that the bithiazole may adjust its mode of binding (i.e., intercalate *or* groove bind) to accommodate DNA structural features flanking the site of metal binding domain association.

In addition to addressing the roles of the metal binding domain and the bithiazole + C-terminus, recent studies have also addressed the role of the disaccharide moiety in DNA recognition and binding. While the overall site-selectivities of Fe(II)·bleomycin and Fe(II)·deglycobleomycin are virtually identical,[161] some studies have shown that Fe(II)·deglycobleomycin and Fe(II)·decarbamoylbleomycin, lacking only the mannose carbamoyl functionality, had significantly altered strand selectivities toward an oligonucleotide substrate, suggesting that the disaccharide plays a role in orientation of binding.[162] More directly, several recent studies have varied systematically the disaccharide moiety while maintaining the remaining portions of bleomycin intact; studies have demonstrated that although exhibiting similar DNA binding affinities, Fe(II)·deglycobleomycin[49,115,116,181] and Fe(II)·decarbamoylbleomycin[146,147,182−184] have similarly diminished cleavage efficiencies and ratios of double-strand to single-strand cleavage relative to intact Fe(II)·bleomycin.

Additionally, another study[181] has also shown that deletion of the terminal mannopyranoside, including the carbamoyl group, produces a monosaccharide analogue with a nearly identical cleavage selectivity and efficiency in comparison to intact Fe(II)·bleomycin. Further, substitution of an α-D-mannopyranose for the α-L-gulopyranose at this now terminal position, while maintaining a similar site selectivity, had a significant negative effect on the overall cleavage efficiency of the drug when compared to intact bleomycin, demannosylbleomycin, and deglycobleomycin.[181] Thus, it has been proposed[181] that while the terminal α-D-mannopyranoside residue has only a small effect upon cleavage activity, the α-L-gulopyranoside residue assists in proper association of the drug to DNA. These findings suggest that although not directly involved in DNA binding, the disaccharide, given its attachment to the metal binding domain, may assist in drug orientation relative to the DNA target strand or groove leading to efficient cleavage. In addition, it appears that the mannose carbamoyl plays little role in promoting selectivity and cleavage efficiency.

The remaining region of bleomycin, the peptide linker, has historically been considered to simply connect the bithiazole DNA-binding group with the metal binding domain; an active and significant role of the linker in the binding or cleavage properties was not thoroughly investigated.[26] However, recent studies of synthetic analogues of this region have indicated that the L-Thr side chain can influence the DNA binding affinity of bleomycin.[115] Further studies[117] in which elements of side chains in the linker region of the complete drug molecule were removed showed that two elements of the linker region directly affected cleavage efficiency: (1) removal of the L-Thr side chain significantly decreased cleavage activity, while (2) removal of the C4-methyl substituent of the 4-aminobutanoic acid subunit of the methyl valerate moiety caused a smaller but still significant reduction in efficiency. In contrast, deletion or stereochemical redirection of the C3-hydroxyl and C2-methyl substituents was not found to have a significant impact on cleavage activity.[117] Though the site selectivity of cleavage was not affected by these changes, the ratio of double-strand to single-strand DNA cleavage was decreased along with overall strand scission efficiency.[117] In another study,[118] *N*-methylation of the L-Thr substituent of deglycobleomycin was found to decrease cleavage efficiency, as with the previously discussed modifications, and to nearly eliminate the characteristic 5′-GT/C site-selectivity.

More recently, two reports were published which sought to specifically delineate the roles of the above two subunits of the linker region.[119,120] In one, systematic variations in the L-Thr side chain (Figure 6) again decreased overall efficiency and the ratio of

double-strand to single-strand cleavage without affecting site-selectivity;[119] results indicated that this substituent within the peptide linker region restricts the number of accessible conformations available and leads to a compact conformation conducive to DNA strand scission. A concurrent study[120] also showed that the C4-Me group contributes significantly to cleavage efficiency and that the C3-hydroxyl does not; this study also demonstrated that the contribution of the C4-methyl group depends on the presence of the C2-methyl group, which by itself has a much smaller effect on cleavage efficiency. In addition, previously discussed results[42,43] demonstrating that the length of the linker region is critical for cleavage activity were also supported in this report. These studies,[119,120] taken together with structural models[18,113,114,185] of free and DNA-bound HOO−Co(III)·bleomycin A_2, point to a critical function of the peptide linker region in which the length and substituents of the region allow the molecule to adopt a compact, optimum preorganized conformation for binding and cleavage prior to actual DNA association. Importantly, the Thr portion of the peptide linker may also create a swivel point[119] to allow the metal binding domain to relocate to an adjacent nucleotide site via one bithiazole interaction leading to double-strand DNA scission. Thus, the length and composition of the peptide linker between the metal binding domain and the bithiazole + C-terminus appears to play a critical role in allowing their "cooperative" interaction with regard to overall efficiency and double-strand cleavage at least at select DNA sites.

4. Direct Studies of Fe·bleomycin–DNA Interactions

While the previously discussed studies have provided indirect evidence of the regions of Fe·bleomycin most involved in DNA recognition and binding, several studies have also attempted to examine more directly changes in the structure of the entire complex, especially the metal binding domain, upon binding to DNA as well as its positioning relative to the DNA substrate. Studies of this type indicate that DNA association leads to changes in the structure of the metal binding domain of Fe·bleomycin; the ESR signal of NO−Fe(II)·bleomycin changes upon binding to calf thymus DNA,[186,187] and the spin state of Fe(III)·bleomycin in phosphate buffer was found to change from high to low upon binding to DNA.[188] These studies suggest that upon DNA binding, metallobleomycin can adopt two different conformations, one significantly altered by the DNA and the other much the same as unbound metallobleomycin; these differences may reflect the binding of metallobleomycin to higher affinity, specific sites on the DNA strand in contrast to nonspecific sites, respectively.

In an additional study addressing the above point,[189] oligonucleotide substrates in lieu of bulk DNA were utilized containing an embedded specific recognition site (5′-GC) or a nonspecific recognition site (5′-AT). As assessed by absorbance and ESR spectroscopies, it was found that various forms of Fe·bleomycin including NO−Fe(II)·bleomycin, $^{\bullet}O_2^{-}$−Fe(III)·bleomycin, Fe(III)·bleomycin, and HOO−Fe(III)·bleomycin all interacted differently with DNA depending upon its nucleotide sequence. While alterations in the structure of each complex were observed upon DNA binding, maximal structural perturbation of the metal binding domain was observed to occur with the oligonucleotide sequence containing the preferred binding site.

In a different study,[190] analysis of hyperfine couplings in Q-band electron nuclear double resonance (ENDOR) spectra between Fe and ^{31}P revealed a well-defined and measurable distance between the Fe center and a DNA phosphate ($\sim$ 7.4 Å) for both Fe(III)·bleomycin and "activated" Fe·bleomycin. Additionally, these spectra have provided indications of possible perturbations of protons within the metal binding domain of bleomycin upon binding to DNA. In another report,[151] changes in the EPR and resonance Raman spectra of Fe(III)·bleomycin were observed upon binding to particular DNA sequences. Specifically, binding to a $d(CG)_3$ oligomer caused a narrowing of the g_{max} portion of the EPR spectrum as well as the Fe−OH band of low-spin Fe(III)·bleomycin, implying a binding-induced conformational restriction of the drug complex. Also observed were differences in the resonance Raman spectra implying conformational changes in the β-hydroxyhistidyl amide, pyrimidine, and axial hydroxide ligands of Fe(III)·bleomycin. Importantly, these changes were only observed upon binding to the $d(CG)_3$ oligomer and not a $d(AT)_3$ analogue.

Overall, the above studies suggest that while recognition of the exocyclic amine of guanine probably occurs as a function of the metal binding domain of bleomycin, this region acts in concert with the other structural moieties of the drug molecule including the bithiazole + C-terminus, peptide linker, and disaccharide to achieve efficient and selective DNA modification. Thus, it is becoming clear that while some functions can be attributed to various regions, metallobleomycin in its entirety must be viewed as producing the final observed outcomes of metal complexation, oxygen activation, DNA recognition, binding, and cleavage. In addition, direct spectroscopic studies seem to imply that the DNA target sequence may alter the structure of metallobleomycin akin to an "induced fit" mechanism of recognition. Hopefully, further studies of this type will continue to shed light directly upon the factors responsible for the site-selectivity of Fe·bleomycin and perturbations in drug structure that occur upon DNA binding which may affect reactivity. While the foregoing studies have added much to our understanding of how Fe·bleomycin recognizes and binds to a target DNA, details concerning the DNA-bound complex can unfortunately only be inferred. Thus, these studies are most valuable when interpreted in light of high-resolution structural studies that have examined metallobleomycin in its entirety which, at present, have only been conducted with metallobleomycins other than Fe·bleomycin.

B. Co·bleomycin

As mentioned in section II.B, Co(II)·bleomycin can be aerobically oxygenated in a fashion reminiscent of Fe(II)·bleomycin to create HOO−Co(III)·bleomycin

and H_2O−Co(III)·bleomycin, the "green" and "brown" complexes of Co(III)·bleomycin, respectively.[19,131,132] In addition, depending upon the conditions employed, several other colored forms of Co(III)·bleomycin can be isolated differing in the nature of the exogenous ligand bound to the metal center, e.g., containing formate or SCN ligands.[26,128] In the case of the hydroperoxy- and aquo-bound Co(III)·bleomycins, the resulting isolable complexes can be photoactivated to cleave DNA through C4′−H abstraction[137] with a selectivity that parallels Fe(II)·bleomycin.[133,138] Thus, oxygenated forms of Co(III)·bleomycin are thought to closely resemble "activated" Fe·bleomycin yet afford reasonably stable,[191] exchange-inert, diamagnetic complexes amenable to NMR structural analyses.[18,19] Additional interest in Co·bleomycin stems from its ability to act as a tumor localization agent through complexation of ^{57}Co and the selective accumulation of the resulting metallodrug in tumor cells.[192,193]

1. Structure

The structures of Co(III)·bleomycin complexes determined prior to 1987 have been reviewed;[26] at that time, two key models for Co(III)·bleomycin were proposed in which (1) the imidazole, deprotonated His amide, pyrimidine, and secondary amine of aminoalanine create equatorial ligands with the primary amine of aminoalanine acting as an apical ligand (leading to a structure similar to that shown for Fe·bleomycin, Figure 3)[194,195] and (2) where the imidazole, pyrimidine, and primary and secondary amines of aminoalanine bind equatorially with the amide nitrogen of aminoalanine acting as an axial ligand;[196] the amide nitrogen of His was not involved in metal binding in this structure.

Recently, information concerning the ligation environment of Co(III)·bleomycin and analogues thereof has been obtained through NMR studies; however, as with other metallobleomycins, some structural ambiguities exist. One study[19,185] compared Co(III)·bleomycin with HOO−Co(III)·bleomycin and proposed the formation of a square pyramidal geometry with equatorial ligation of the secondary amine, pyrimidine, amide, and imidazole with the primary amine occupying the axial position for both forms. Interestingly, several NOEs were observed for both the five- and six-coordinate complexes which indicated folding of the peptide linker region. Molecular modeling and energy minimization of these structures based on NOE constraints led to a proposed overall conformation in which the bithiazole region is folded underneath the metal center of HOO−Co(III)·bleomycin in a compact arrangement; this association of the bithiazole with the metal binding domain was not observed for the five-coordinate complex.

A further study[191] that specifically examined the structure of isolated HOO−Co(III)·bleomycin in detail supported the above ligand arrangement and compared four possible ligation isomers in which the axial ligand was either the primary amine of aminoalanine or the mannose carbamoyl, with either possible screw sense for each. The validity of each

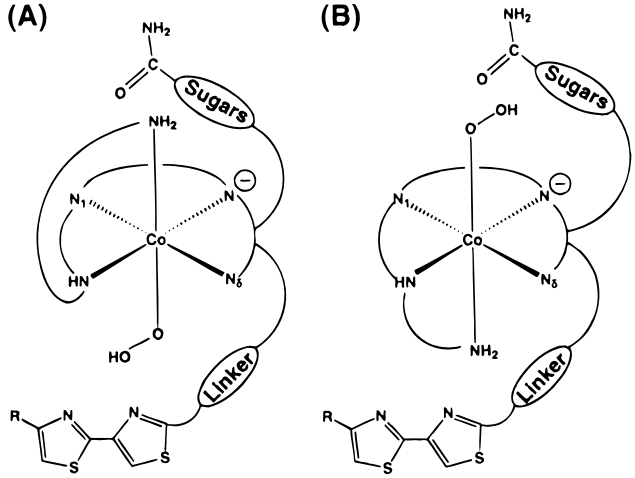


Figure 7. Structures of HOO−Co(III)·bleomycin in two screw senses with the primary amine of aminoalanine as an apical ligand. Structure A is favored in these studies. (Reprinted with permission from ref 198. Copyright 1998 American Chemical Society.)

model was evaluated via molecular dynamics calculations based on NMR data. Ultimately, this study also favored axial ligation of the primary amine and additionally specified a particular screw sense in the complex formed (Figure 7). The structural model proposed in this study again suggested that the bithiazole moiety was folded beneath the metal binding domain. Later examination of Co(III)·bleomycin "brown"[197] and Co(III)·deglycobleomycin "green"[198] concluded that an identical ligand composition and arrangement was present in these alternative forms of Co(III)·bleomycin. In contrast to the above, however, a study[199] of HOO−Co(III)·pepleomycin, an analogue of bleomycin containing a C-terminal phenyl substituent, and HOO−Co(III)·deglycopeplomycin proposed that the carbamoyl nitrogen is the preferred axial ligand based upon what the authors suggested was a more complete assignment of exchangeable protons. It was also found from these studies[199] that the primary amine occupies the axial site only in the case of the deglycosylated pepleomycin when the carbamoyl nitrogen is no longer present in the structure for ligation.

Despite the differences between bleomycin and pepleomycin, these studies suggest distinct similarities in the overall structures of their metal binding domains. As with Fe·bleomycin, it appears that the carbamoyl nitrogen may participate in some level of metal complexation but the factors that influence it may be rather subtle. Aside from this issue, however, the above studies indicate that oxygenated Co(III)·bleomycin may exhibit many structural parallels to the activated Fe·bleomycin complex, including its ligand arrangement and oxygenation. These features were exploited in understanding the structure of DNA-bound Co·bleomycin as described in the following section.

2. DNA Recognition and Binding

Recently, NMR studies have been performed which have provided detailed information concerning the structure of Co(III)·bleomycin bound to oligonucleo-

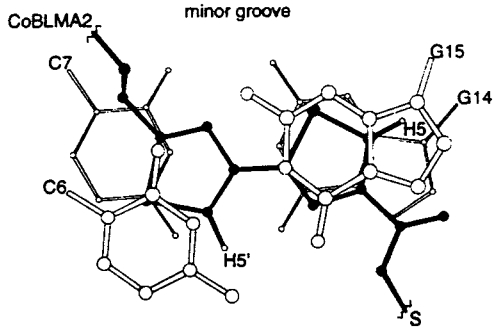


Figure 8. Illustration of bithiazole (dark) partial DNA intercalation viewed down the DNA helical axis. The terminal thiazole ring is stacked between two guanine nucleobases (G_{14} and G_{15}), while the penultimate thiazole ring is partially stacked between two pyrimidine bases (C_6 and C_7). (Reprinted with permission from ref 197. Copyright 1997 American Chemical Society.)

tide substrates. Examination of HOO−Co(III)·bleomycin "green" associated with the self-complementary oligonucleotide d(CCAGGCCTGG)$_2$, which contains a specific recognition and cleavage site at C_6, led to the first model[113,200] of this metallobleomycin positioned within the DNA minor groove based on the observation of 60 intermolecular NOEs (for models of these structures see refs 18 and 30). Partial intercalation of the bithiazole region into the DNA helix (Figure 8), leading to a 13° unwinding angle, was proposed to occur between the C_6·G_{15} and C_7·G_{14} base pairs of this oligonucleotide based upon intermolecular NOEs and observed upfield shifts in both bithiazole protons upon binding to DNA; full stacking occurred between the terminal thiazole ring and G_{14} and G_{15} while the penultimate thiazole ring only partially stacked between C_6 and C_7. With this arrangement the terminal dimethylsulfonium extends through the DNA helix and resides in the major groove of the oligonucleotide. Additional NMR studies[201] were performed with this metallobleomycin bound to d(GGAAGCTTCC)$_2$ and d(AAACGTTT)$_2$, which provided 5′-GPy sites with significantly different flanking sequences and demonstrated intercalation at the C_6−C_7 and T_6−T_7 regions, respectively. Thus, sequence-specific bithiazole intercalation was not observed to occur within the complexes formed. Additionally, a limited number of NOEs were observed between metal binding domain protons and G_5 in each oligonucleotide examined.[201]

Importantly, the above complexes revealed[18,113] a plausible basis for metallobleomycin 5′GC/T site-selectivity in the form of hydrogen bonds between the 4-amino group and N3 of the bleomycin pyrimidine moiety and the N-3 and exocyclic 2-amino group of G_5 of d(CCAGGCCTGG)$_2$, respectively (as also supported through studies of synthetic bleomycin analogues[30]); this arrangement forms essentially a triplex with the purine·pyrimidine (G·C) Watson−Crick base pair of the DNA target and the pyrimidine of bleomycin (Figure 9). Additionally, the formation of this triplex was proposed to increase the strength of metallobleomycin binding to DNA, thus possibly allowing Fe·bleomycin reactivation to occur, as described in previous sections, facilitating double-strand DNA cleavage. In addition to the above,

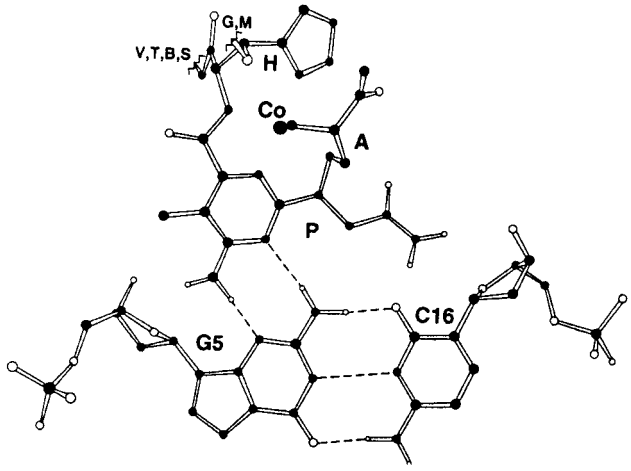


Figure 9. Formation of a base triplex at the site of metallobleomycin binding to 5′-GPy sites. The pyrimidine moiety (P) of bleomycin forms a minor groove interaction with the G_5·C_{16} base pair via hydrogen bonds between the 4-amino group and N3 of P and N3 and 2-amino group of G_5. (Reprinted with permission from ref 197. Copyright 1997 American Chemical Society.)

essentially identical structures for DNA drug complexes involving Co(III)·bleomycin analogues, in terms of both bithiazole intercalation and positioning of the pyrimidine group within the minor groove, were produced in several studies, including HOO−Co(III)·bleomycin complexed with d(CCAGTACTGG),[114] d(GGAAGCTTCC), and d(AAACGTTT)[201] and of H_2O−Co(III)·bleomycin "brown" with d(CCAGGCCTGG).[197] Also, despite reported differences in their metal chelation as mentioned in the previous section, the above studies of Co(III)·bleomycin and Co(III)·pepleomycin[202] both propose that the bithiazole moiety partially intercalates DNA and that hydrogen bonding occurs between the pyrimidine moiety and minor groove features of the G residue proximal to the intercalation site.

With parallels to intact Co(III)·bleomycin, examination of a model of Co(III)·bleomycin, AMPHIS−NET, complexed with d(CGCAATTGCG)$_2$ provided further evidence of minor groove binding[203] and also led to early evidence that the solvent coordination site of Co is directed into the minor groove, in agreement with the proposed nondiffusion-based mechanisms of strand cleavage by metallobleomycin. This initial view was supported and refined in the study of Co(III)·bleomycin "green"[113] described above which located the hydroperoxide ligand near the C4′−H of C_6 in d(CCAGGCCTGG)$_2$ via NOEs between this ligand and minor groove protons of C_6 and C_7, thus providing the opportunity for C4′−H abstraction by this ligand. More detailed NMR examination[204] of the above model compound, AMPHIS−NET, bound to d(CGCAATTGCG)$_2$ revealed NOE interactions between the pyrimidine H8 and the H4′ of C_9 and between the imidazole H2 and H4′ of A_{15}, thus further demonstrating a basis for recognition and cleavage at d(GpPy) sites within this model compound.

Although the preceding studies did not propose a role for the disaccharide region in DNA binding or cleavage, subtle differences were seen in the NMR

spectra of HOO−Co(III)·deglycobleomycin with d(CCAGGCCTGG)[198] in comparison to intact Co(III)·bleomycin which implicate the disaccharide in nonspecific DNA interactions which could result in enhanced cleavage efficiency. "Minor complexes" of the deglyco form with DNA were observed which were not seen in previous studies of the intact drug; the proposed model thus includes secondary binding sites, one of which leads to a cleavage event. The equilibrium between this active secondary site and the primary site also corresponds to the observed decrease in binding affinity for deglycobleomycin relative to the intact drug. Additionally, greater conformational flexibility was observed in the T_{18}·A_3 base pair in the deglycobleomycin·oligonucleotide complex; previous models of the intact drug·DNA complex suggest that the disaccharide region is positioned adjacent to this base pair, constraining flexibility at this site. Thus, the disaccharide is seen to contribute primarily to binding affinity via conformational stability of the drug·DNA complex rather than to binding or cleavage selectivity, in agreement with studies of synthetic modifications of this region as discussed in detail in section III.A.3.

The above studies of the Co·bleomycins have added much to our knowledge of metallobleomycin in general including a view of the metallodrug structure alone as well as bound to a DNA substrate. These studies indicate that the metallodrug is likely to be structurally preorganized prior to DNA binding and that upon DNA association, the metal binding domain precisely interacts with the G·C base pair of the 5′-GPy of preferred sites leading to a productive orientation of the oxidizing moiety of the drug relative to its C4′−H target. In addition, (1) the linker peptide promotes this productive orientation and also allows the bithiazole to partially intercalate 3′ to the site of nucleotide modification while (2) the disaccharide assists in providing nonspecific contacts leading to a more precise positioning of the metallodrug relative to its DNA target.

C. Zn·bleomycin

Diamagnetic Zn(II)·bleomycin has been used as a structural model of Fe(II)·bleomycin for a number of years[26,41] in order to produce a metallobleomycin amenable to NMR structural techniques. Despite its lack of redox activity and inability to mediate DNA strand scission, useful structural information has been inferred from the use of this metallobleomycin analogue; the ligand lability and dynamic nature of this metallobleomycin may perhaps serve to mimic the behavior of Fe(II)·bleomycin prior to its oxygenation which evidence suggests, as discussed in section III.A.3, is a labile structure that can be altered upon DNA association.

1. Structure

Early studies[205−207] of Zn(II)·bleomycin led to a proposed structure,[146,147] paralleling CO−Fe(II)·bleomycin, in which the pyrimidine, imidazole, and β-aminoalanine primary and secondary amines were thought to be ligated to the metal equatorially with the mannose carbamoyl acting as an axial ligand.

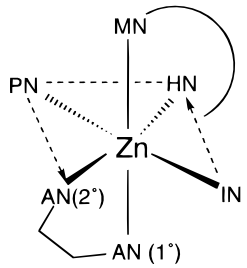


Figure 10. Structure of the ligand environment of Zn(II)·deglycobleomycin with the preferred screw sense as determined by examination of intra- and intermolecular NOEs upon oligonucleotide binding. AN(1°) and AN(°2), PN, HN, IN, and MN are the primary and secondary amines of aminoalanine, pyrimidine nitrogen, His amide, His imidazole, and mannose carbamoyl nitrogens, respectively. (Adapted with permission from ref 213. Copyright 1998 American Chemical Society.)

Later attempts at comprehensive ^{1}H and ^{13}C NMR studies[208−210] of Zn(II)·bleomycin provided stronger evidence that the β-aminoalanine secondary amine, β-hydroxyhistidine amide, imidazole, pyrimidine, and mannose carbamoyl are coordinated to the Zn(II) center reportedly without a regular coordination symmetry.[208] In addition, another study using ^{113}Cd·bleomycin[211] demonstrated that this complex had nearly identical ^{13}C chemical shifts relative to those of Zn(II)·bleomycin, thus implying a similar structure, and allowed direct observation of an equilibrium between different coordination environments which could be varied in a temperature-dependent fashion.

In addition to bleomycin, the Zn(II) complex of tallysomycin,[212] a naturally occurring congener of bleomycin, was shown to exhibit identical ^{1}H and ^{13}C NMR shifts and NOESY spectra to those of Zn(II)·bleomycin. This report also established binding of the same ligands as those described in previous studies but excluded carbamoyl binding to the metal center. Further, this study also more closely examined the arrangement and chirality of the ligands involved in metal binding; NMR results, in combination with molecular dynamics calculations, led these authors to suggest an alternative Zn(II) coordination involving the primary and secondary amines of β-aminoalanine, imidazole, and His amide equatorially with the pyrimidine nitrogen acting as an apical ligand.

While the studies discussed above suggest structural differences between Zn(II)·bleomycin and the metallobleomycin of likely therapeutic relevance, more recent results, involving molecular dynamics calculations performed in conjunction with intramolecular and intermolecular NOEs derived from the examination of d(CGCTAGCG)$_2$-bound Zn(II)·deglycobleomycin A_2,[213] support coordination of the imidazole, β-hydroxyhistidine amide, pyrimidine, and secondary amine of β-aminoalanine equatorially with the primary amine acting as an apical ligand, resulting in a structure similar to that shown for Fe·bleomycin (Figure 3). Importantly, intermolecular NOEs between the bound complex and the DNA substrate dictated a preferred screw sense within the complex as shown in Figure 10.

Overall, these studies again indicate that as with other metallobleomycins, ambiguities exist in our

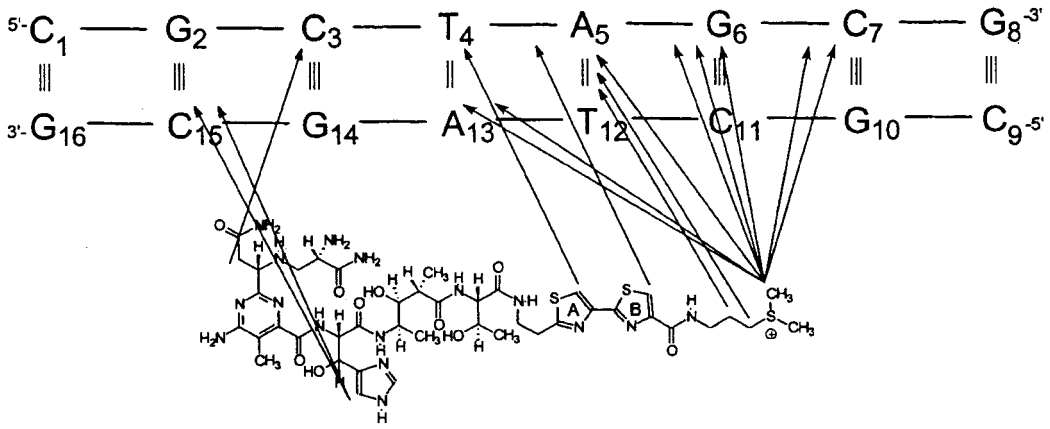


Figure 11. Illustration of the intermolecular NOEs, represented by arrows, between Zn(II)·deglycobleomycin and d(CGCTAGCG)$_2$. (Reprinted with permission from ref 213. Copyright 1998 American Chemical Society.)

understanding of Zn(II)·bleomycin structure. No doubt, the labile nature of Zn(II) contributes to the difficulty in achieving an exact consensus with this particular metallobleomycin. However, many similarities exist between the structures proposed and other metallobleomycins which may parallel the labile nature of unoxygenated Fe(II)·bleomycin.

2. DNA Recognition and Binding

A series of recent NMR studies have examined the structures of Zn(II)·bleomycins bound to DNA substrates using a variety of multidimensional NMR techniques in combination with molecular dynamics calculations. Specifically, the octanucleotide d(CGCTAGCG)$_2$ was selected in these studies based upon its usefulness as a selective cleavage substrate for Fe·bleomycin.[178] An initial communication[214] reported results utilizing Zn(II)·bleomycin A_5, in which the C-terminal sulfonium is replaced by a spermidine moiety. In this study, binding of Zn(II)·bleomycin in the minor groove of DNA was supported by both NOESY data and molecular dynamics calculations; however, the exact binding mode of the bithiazole and C-terminal substituent remained ambiguous, since the data supported both minor groove binding and partial intercalation. Interestingly, DQF−COSY measurements indicated that the oligonucleotide did not deviate significantly from its native B-form conformation upon binding of bleomycin, indicating that intercalation was not likely.

Later, further examination[215] and refinement of this model was accomplished through additional NMR studies of Zn(II)·bleomycin A_5 and A_2 complexed to this same octanucleotide to yield eight intermolecular NOEs; NOEs were found between DNA minor groove protons and the bithiazole along with the metal binding domain. Following molecular dynamics calculations, a structural model was proposed involving the partial intercalation of the bithiazole moiety into the helix at the A_5G_6·C_3T_4 step inducing a bend in the DNA and satisfying the observed upfield shifts of the bithiazole aromatic protons. Further, the Zn(II)·bleomycin complex was shown to reside primarily in the minor groove as a folded structure, with both bithiazole hydrogens directed into the groove in a *cis*-orientation. The final model (see ref 215 for a detailed depiction of this model) resulting from molecular dynamics also suggested the formation of a hydrogen bond between the exocyclic amine of guanosine and the methyl valerate hydroxyl group, an interaction that could be involved in DNA sequence recognition by metallobleomycin.

Most recently,[213] examination of Zn(II)·deglycobleomycin A_2 complexed with d(CGCTAGCG)$_2$ demonstrated exclusive minor groove binding of the bithiazole and C-terminus within this alternative bleomycin analogue; upfield shifts of both bithiazole protons, expected upon partial intercalation, were not observed in this study. Additionally, no loss of intrastrand NOE interactions was observed, indicating that the distance between stacked base pairs was not altered upon drug binding. Rather, new NOEs were observed indicating interactions between minor groove protons and the sulfonium C-terminus of bleomycin. Modeling based on 16 intermolecular NOEs (Figure 11) and molecular dynamics calculations placed the C-terminus of Zn(II)·deglycobleomycin within the central T·A region with DNA binding occurring in a lexitropsin-like fashion (Figure 12)[216−218] and the metal binding domain positioned to interact with its preferred site of nucleotide modification, C_3 (as also supported by the recent study of controlled pore glass conjugated Fe(II)·deglycobleomycin[180] and the cleavage of the same oligonucleotide substrate with Fe(II)·deglycobleomycin possessing a chlorobithiazole[177]). The above modeling (see ref 213 for a detailed depiction of this model) thus positioned the bithiazole 3′ to the cleavage site of this oligonucleotide, suggesting that the bithiazole + C-terminus may substantially contribute to recognition of the DNA sequence flanking the actual metal-induced cleavage site. Unfortunately, while these data focused on the interaction of the bithiazole, a detailed examination of the precise contacts between the metal binding domain relative to its 5′-GC_3 target were not obtained.

Overall, the above observations with Zn(II)·bleomycin and Zn(II)·deglycobleomycin led the authors to suggest as a working hypothesis that the bithiazole may be a versatile DNA binding agent, like other nonfused aromatic ring systems[219,220] it may alter its mode of DNA binding depending upon the sequence encountered 3′ to the metal binding domain. Thus, while in fast exchange, it is still interesting to

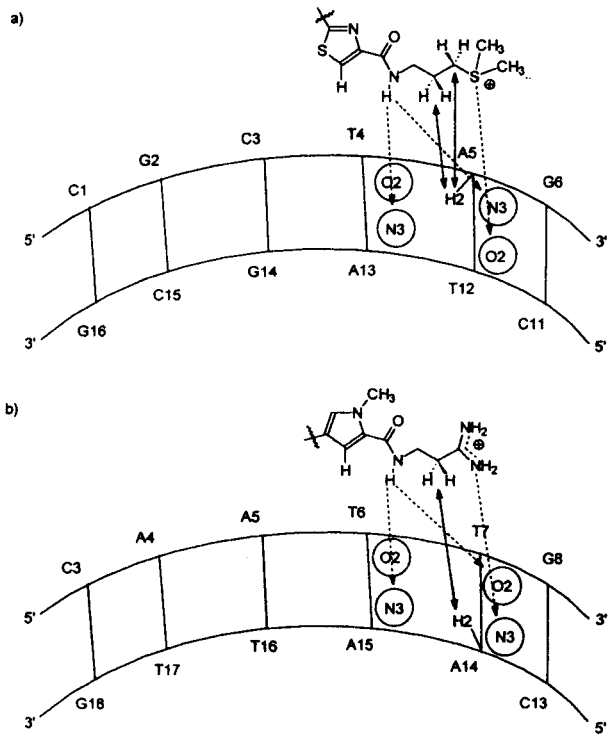


Figure 12. Comparison of DNA minor groove binding by (a) the bithiazolecarboxamide moiety of bleomycin and (b) the *N*-methylpyrrolecarboxamide of lexitropsins. Hydrogen bonds and electrostatic interactions (dashed arrows) and NOEs (solid arrows) formed upon DNA binding are indicated. (Reprinted with permission from ref 213. Copyright 1998 American Chemical Society.)

speculate that the bithiazole−DNA interaction within Zn(II)·bleomycin may perhaps participate in a continuum of DNA-bound structures from groove binding at one extreme through intercalation at the other extreme; these modes of binding may be influenced by the nature of the metal binding domain and its DNA-bound structure or the sequences flanking a DNA target site. As the many biophysical studies of the bithiazole may attest, there is certainly adequate evidence to support the existence of all these binding modes as discussed in section III.A.3.

IV. Summary

While many details of metallobleomycin structure and DNA recognition have yet to be determined, the foregoing studies have served to advance considerably our knowledge of the topic. The studies described above are beginning to shed light on some level of generalization concerning the functional features of metallobleomycin including the role of various domains and their activities. In general, the metal binding domain forms transition-metal complexes with four equatorial nitrogenous ligands from the imidazole of β-hydroxyhistidine, the deprotonated amide nitrogen of His, the pyrimidine ring nitrogen, and the secondary amine of β-aminoalanine. In addition, the primary amine of β-aminoalanine most likely binds axially within this structure with the remaining axial coordination site occupied by the carbamoyl nitrogen or dioxygen as in activated bleomycin. This domain, which appears to be a dynamic structure both in the absence and presence of DNA, is also central to the activation of oxygen, 5′-GT/C sequence selectivity, and the precise orientation of a metal-bound oxidizing equivalent with relation to the C4′−H of a target nucleotide.

Meanwhile, the bithiazole + C-terminus of bleomycin is required for proper DNA association of metallobleomycin and may also contribute to the total selectivity of the drug−DNA interaction; while studies of models of activated metallobleomycin have shown that the bithiazole has the ability to intercalate, groove bind, or partially intercalate DNA, threading intercalation may not be an absolute requirement for Fe(II)·bleomycin to exhibit DNA cleavage and its characteristic site selectivity. These and past studies of the bithiazole suggest that the bithiazole may be a versatile DNA binder with the ability to alter its mode of DNA interaction in a fashion dependent upon the sequence of DNA flanking the 3′-side of a cleavage site occupied by the metal binding domain of metallobleomycin. Thus, future studies should seek to determine the preferred sites of minor groove binding or intercalation and correlate these to locations of double-strand DNA scission.

In addition to the above two functional domains of metallobleomycin, the methylvalerate−Thr linker peptide appears vital to DNA cleavage efficiency and the ability to generate double-strand lesions. This linker may also contribute to DNA site-selectivity and to the generation of a folded metallobleomycin structure that is preorganized for DNA binding yet possibly capable of allowing the metal binding domain to relocate to an adjacent nucleotide site. Along with the linker, the disaccharide moiety appears to contribute to nonspecific DNA binding interactions leading to a more conformationally restricted DNA complex, metal complexation, and the overall efficiency of DNA cleavage.

Overall, the bleomycins are complex natural products that have evolved to permit the functional domains of these antibiotics to work cooperatively toward efficient DNA binding and strand scission. Thus, while many elegant studies have sought to dissect the molecule into its component parts leading to insight into their functions, bleomycin in its entirety needs to be viewed as leading to the final outcome of its activity. Hopefully, current and further study of the activity of the metallobleomycins, as aided by improved total syntheses,[30,221] will seek to explore the cooperativity that occurs between the domains of bleomycin in an attempt to understand these natural products fully and to mimic their behavior in analogues with potentially increased clinical activity.

V. Acknowledgments

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