An Investigation of the Interaction of DNA With Selected Peptides and Proteins

by

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ABSTRACT

The communication of genetic material with biomolecules has been a major interest in cancer biology research for decades. Among its different levels of involvement, DNA is known to be a target of several antitumor agents. Additionally, tissue specific interaction between macromolecules such as proteins and structurally important regions of DNA has been reported to define the onset of certain types of cancers.

Illustrated in Chapter 1 is the general history of research on the interaction of DNA and anticancer drugs, most importantly different congener of bleomycin (BLM). Additionally, several synthetic analogues of bleomycin, including the structural components and functionalities, are discussed.

Chapter 2 describes a new approach to study the double-strand DNA lesion caused by antitumor drug bleomycin. The hairpin DNA library used in this study displays numerous cleavage sites demonstrating the versatility of bleomycin interaction with DNA. Interestingly, some of those cleavage sites suggest a novel mechanism of bleomycin interaction, which has not been reported before.

Cytidine methylation has generally been found to decrease site-specific cleavage of DNA by BLM, possibly due to structural change and subsequent reduced bleomycin-mediated recognition of DNA. As illustrated in Chapter 3, three hairpin DNAs known to be strongly bound by bleomycin, and their methylated counterparts, were used to study the dynamics of bleomycin-induced degradation of DNAs in cancer cells. Interestingly, cytidine methylation on one of the DNAs has also shown a major shift in the intensity of
bleomycin induced double-strand DNA cleavage pattern, which is known to be a more potent form of bleomycin induced cleavages.

DNA secondary structures are known to play important roles in gene regulation. Chapter 4 demonstrates a structural change of the BCL2 promoter element as a result of its dynamic interaction with the individual domains of hnRNP LL, which is essential to facilitate the transcription of BCL2. Furthermore, an in vitro protein synthesis technique has been employed to study the dynamic interaction between protein domains and the i-motif DNA within the promoter element. Several constructs were made involving replacement of a single amino acid with a fluorescent analogue, and these were used to study FRET between domain 1 and the i-motif, the later of which harbored a fluorescent acceptor nucleotide analogue.
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</tr>
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<td>Adenosine-5’-monophosphate</td>
</tr>
<tr>
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<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BLM</td>
<td>Bleomycin</td>
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<tr>
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</tr>
<tr>
<td>µCi</td>
<td>Micro Curie</td>
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<tr>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per Minute</td>
</tr>
<tr>
<td>Cy5</td>
<td>Potassium 2-((1E,3E,5E)-5-(1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3Hindol-1-ium-5-sulfonate</td>
</tr>
<tr>
<td>Cy5**</td>
<td>Potassium 2-((1E,3E,5E)-5-(1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-3-methyl-1,3-bis(4-sulfobutyl)-3H-indol-1-ium-5-sulfonate</td>
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<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
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<td>Ethyl Acetate</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
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<td>Normal</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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</tbody>
</table>
nt  Nucleotide
nm  Nanometer
NMR Nuclear Magnetic Resonance
NOE Nuclear Overhauser Effect
PBS Phosphate Buffered Saline
pdCpA 5''-O-phosphoryl-2''-deoxycytidylyl(3''→5'')adenosine
PTC Peptidyltransferase Center
pYRNA8 Plasmid Containing the Gene Encoding 74-nucleotide tRNA_{CUA}
RNA Ribonucleic Acid
rt Room Temperature
SDS–PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
soln Solution
SPR Surface Plasmon Resonance
TAE Tris-acetic Acid-EDTA
TBE Tris-borate-EDTA
Tris Tris(hydroxymethyl)aminomethane
tRNA Transfer RNA
Trp Tryptophan
UV Ultraviolet
CHAPTER 1
GENERAL INTRODUCTION

1.1. Molecular Therapeutics of Cancer Research

Cancer is an uncontrolled growth of normal cells in the body. It claims millions of lives worldwide every year and it is a major public health concern.\(^1\) Symptoms of cancer involve abnormal cell growth, bleeding, cough, and weight loss.\(^2\) Among several types of cancers, lung and prostate cancers are most common in men, whereas breast, cervical and ovarian cancers are prevalent in females.\(^3\) Leukemia, brain tumors and skin cancers are other types of cancer that affect a wide population of all ages.\(^4\) Increased risk of cancer is associated with developing countries\(^5\) and with age,\(^6\) though it has been argued that the increased life expectancy has contributed to the higher occurrence of cancer in developed countries. Although the majority of cancers most likely develop due to environmental causes,\(^7\) genetics\(^8\) and lifestyle also play an important role in the development of malignancies. Other factors include smoking,\(^9,10\) diet, infection, exposure to radiation and stress.\(^11\) The treatment of cancer is practiced at several different levels, including chemotherapy, radiation therapy, hormonal treatment and patient counseling.

Chemotherapy research broadly involves the synthesis and study of novel chemotherapeutic agents for the diagnosis and treatment of cancer. Therapeutic anticancer agents are mainly designed with a curative intent to prolong life. DNA-targeted agents constitute a broad class of therapeutic anticancer agents and can be classified into sub-categories such as, intercalators and groove binders (Figure 1.1).
Figure 1.1. Groove Binding of Hoescht 33258 to the Minor Groove of DNA (A, NDB Structure 8bna)\textsuperscript{12} and the Intercalation of Ellipticine into DNA (B, NDB Structure 1z3f).\textsuperscript{13}

DNA intercalation involves insertion of a planar molecule in between base pairs, which results in helix unwinding while groove binders are mainly crescent-shaped molecules, which interact with the minor groove of DNA. Without inducing large conformational changes in DNA, these groove binders have higher association energy than intercalators and they are of high clinical utility as therapeutic antitumor agents.\textsuperscript{14} Some of the classical DNA intercalators and groove binders are listed in Figure 1.2.
1.2. Bleomycin and DNA

Bleomycins are a class of DNA minor groove binding or intercalative glycopeptide antibiotics, known for their antitumor activity. The cytotoxicity of bleomycin is believed to result from sequence-selective oxidative cleavage of DNA\textsuperscript{16,17} and RNA\textsuperscript{18} in the presence of oxygen and metal ion cofactors. In nature, bleomycin is produced by \textit{Streptomyces verticillus}. In 1966, Umezawa and co-workers extracted bleomycin from \textit{S. verticillus} in the copper-chelated form, and extensively studied its antimicrobial activity\textsuperscript{19,20}. The drug was marketed in Japan by Nippon Kayaku in 1969. In July 1973, bleomycin gained FDA approval and was marketed as an antitumor agent by Bristol Laboratories, under the trade name Blenoxane. Currently, in the United States the drug is being used in combination with other antitumor agents. The main components of Blenoxane are different congeners of bleomycin, primarily BLM A\textsubscript{2} (~60\%) and BLM B\textsubscript{2} (~30\%) (Figure 1.3). In other parts of the world, bleomycin A\textsubscript{5} (pingyangmycin) is
more widely used. In China, bleomycin A₅ has largely replaced bleomycin A₂ because of its relatively low cost of production and it is used for the treatment of a larger variety of cancers including breast and liver. Pingyangmycin (bleomycin A₅) is also known for causing less damage to the lungs during chemotherapy, although prolonged treatment can cause pulmonary fibrosis. Unlike the other variants of bleomycin, BLM A₅ can trigger anaphylactic shock and can be fatal even at low dosage.

Figure 1.3. Different Congeners of Bleomycin.

The tumor cell killing effect of bleomycin has been largely attributed to oxygen mediated DNA cleavage by bleomycin. Therefore oxygen rich pulmonary tissues are believed to be more susceptible to bleomycin induced damage. A different hypothesis
states that the polyamide side chains of bleomycin are the reason of toxicity in lung tissues. Similar phenomena are also observed with other structurally similar glycopeptide antibiotics, such as phleomycin, zorbamycin and tallysomycin (Figure 1.4).\textsuperscript{25-27}

**Figure 1.4.** Structural Compositions of Antitumor Antibiotics Bleomycin A\textsubscript{2}, B\textsubscript{2}, Tallysomycin S\textsubscript{10}B, Phleomycin D\textsubscript{1} and Zorbamycin.\textsuperscript{26}

1.3. **Structural Domains of Bleomycin**

The total synthesis of bleomycin was achieved in the Hecht\textsuperscript{28} and Umezawa\textsuperscript{29} laboratories in 1982 and in the Boger laboratory\textsuperscript{30} in 1994 followed by the successful synthesis of a library of deglycobleomycins in the Hecht laboratory in 2003 using solid phase synthesis.\textsuperscript{31} As illustrated in Figure 1.5, each of the structural domains of
bleomycin participates in key interactions responsible for the efficient binding and cleavage of DNA. Four different subunits of bleomycin include a disaccharide subunit, a metal binding domain, a linker region, and a C-terminal DNA binding domain, the latter of which typically includes a positively charged aliphatic subunit.

**Figure 1.5.** Schematic of HOO-Co(III)•Bleomycin B2 Highlighting the Metal Binding Domain, Linker Region, Disaccharide Moiety and C-Terminal Substituent. Atoms involved in H-bonding to the DNA are in red, and atoms involved in intramolecular H-bonding are in blue.  

The disaccharide subunit of bleomycin, composed of L-gulose and D-mannose, has been studied for its contribution to cellular recognition and uptake (Figure 1.6).  

6
Recently the Hecht laboratory reported that bleomycin and its carbohydrate moiety, conjugated to a cyanine dye, bound selectively to cancer cells.\textsuperscript{34} It was also shown that the carbamoyl functionality in the disaccharide moiety was required for tumor cell targeting.\textsuperscript{34}

![Diagram of bleomycin A₅ and carbohydrate moiety](image)

**Figure 1.6.** Carbohydrate Moiety of Bleomycin. (a) Structure of BLM A₅, in which the highlighted domain shows the carbamoylmannose. (b) Structures of BLM monosaccharide bound to Cy5** (1), decarbamoyl BLM moiety monosaccharide bound to Cy5** (2), and the BLM monosaccharide–Cy5** trimer (3).\textsuperscript{34}

The metal binding domain, consisting of a pyrimidoblastic acid and a β-hydroxyhistidine moiety, is known to interact with and activate first row transition metal
ions, such as Fe, Cu, Co, Mn, Ni, Ru, and V. Recently, in a computational modeling based study, Buda et al. described pyrimidoblamic acid as the primary metal chelating domain of bleomycin (Figure 1.7). These specific domains are also key factors for the site-specific binding of bleomycin-DNA interaction (Figure 1.8).

**Figure 1.7.** Metal Chelating Domain of Fe(II)•BLM.

The bleomycin linker region consists of methylvalerate and L-threonine moieties and is responsible for preorganization of the molecule for DNA cleavage. Early studies in the Boger and Hecht laboratories demonstrated that an altered linker region in bleomycin confers less sequence selectivity of DNA cleavage and dramatically decreases DNA cleavage efficiency. In a similar study, Hecht and coworkers confirmed that the length and nature of the methylvalerate domain of bleomycin also play key roles in determining the DNA cleavage efficiency of bleomycin.
Figure 1.8. Interactions of the Metal–Binding Domain and Disaccharide Moiety With DNA. (A) Stereodiagram of the minor groove of DNA (gray) shown with the metal–binding (yellow with Co in green) and disaccharide (purple) domains and a modeled peroxide ligand (cyan) as a ball-and-stick model. Hydrogen bonds are represented as dashed lines with interacting O atoms in red and N atoms in blue. Intermolecular bonds are black, intramolecular bonds are blue, and magenta bonds represent interactions of the propionamide as modeled (the propioanamide is absent in electron density). The red stick indicates the connection to the linker domain. (B) Schematic showing the hydrogen bonding interactions of the metal–binding and disaccharide domains in the minor groove with bonds colored as in A.32

The DNA binding domain of bleomycin has a C-terminal substituent and a bithiazole moiety, which interact with DNA and binds to the minor groove, helping the
bleomycin molecule to position itself against DNA backbone. In 1985 Henichart et al. employed EPR, fluorescence and viscometry techniques with synthetic 2',4-disubstituted bithiazoles, structurally related to the "tripeptide S" moiety of bleomycin. They reported a partial intercalation of the ring between the base pairs. Moreover, a slight decrease of DNA length in the presence of bithiazole derivative was also revealed, which led to the introduction of a binding model that partially inserts of a thiazole ring in between the bases at a binding site of DNA (Figure 1.9).

![Figure 1.9. Proposed Model for the Insertion of the Bithiazole Moiety of Bleomycin into the DNA Kink.](image)

In an effort to further understand the interaction between bithiazole and DNA, several bithiazole analogues were synthesized in the Hecht laboratory for their incorporation into deglycobleomycin (Figure 1.10). It was concluded that an intact bithiazole moiety is essential for DNA binding and subsequent cleavage. A study
in the Hecht laboratory also demonstrated that a trithiazole moiety (Figure 1.11) had undergone cleavages at less predominantly observed 5’-GT-3’ sequences whereas the bithiazole moiety was more selective for 5’-GC-3’ sequences.

**Figure 1.11.** Deglycobleomycin With a Trithiazole Moiety.

### 1.4. Pathway of DNA Cleavage by Bleomycin

The activation of bleomycin results in a species containing BLM and a metal-oxo complex and leads to DNA cleavage by two different pathways (Figure 1.12).
After activation bleomycin initiates DNA cleavage in a sequence-selective manner. The process is initiated by the abstraction of the C4’ hydrogen atom of a pyrimidine situated to the 3’-side of a deoxyguanosine. Depending on the availability of oxygen this radical can mediate DNA cleavage via two different pathways. The frank strand scission is observed in presence of oxygen where oxygen reacts with the formed radical to undergo a C4’-peroxy radical based Criegee-type rearrangement, which results in a DNA lesion with a 3’-phosphoglycolate, an oligomer with a 5’-phosphate and a base propenal. In

**Figure 1.12.** Pathways of DNA Cleavage by Bleomycin.
contrast, the “anaerobic” pathway leads to a transformation producing a 4’-hydroxyapurinic acid moiety and the release of a nucleobase. This abasic site yields an oligonucleotide terminating in a 3’-phosphate or 3’-hydroxycyclopentenone moiety, when treated with alkali.\textsuperscript{44-48}

The degradation of DNA by bleomycin is a complex, multistep process and has been a subject of ongoing debate over the past few decades. It is postulated that the Fe in Fe•bleomycin can cycle repeatedly through Fe(II) and Fe(III) to facilitate a steady activation of bleomycin. Research has shown that superoxide and peroxides, as a byproduct of frank strand scission, contribute to the process of Fe•BLM reactivation (Figures 1.13 and 1.14).\textsuperscript{17,49,50}

**Figure 1.13.** Possible Activated Fe•Bleomycin Formation Pathways.\textsuperscript{17}
In my research, a combination of a low concentration of bleomycin, comparable to the clinically approved dosage, and a library of hairpin DNAs that strongly bind to bleomycin were employed to investigate the biochemistry of bleomycin A5 induced double-strand DNA cleavage.
CHAPTER 2
MECHANISM OF BLEOMYCIN INDUCED DOUBLE-STRAND DNA CLEAVAGE

2.1. Introduction

Even though it is clear from early studies that the cytotoxicity of bleomycin is a result of its DNA cleavage activity, and the single-strand mode of cleavage is well understood, the nature of DNA double-strand breakage has not been as well characterized. In DNA cleavage by bleomycin, a secondary coupled cleavage has been observed at the site on the opposite strand directly opposing the primary lesion, occurring at the frequency of 20% of the total DNA cleavage.

For primary cleavage sites containing a 5′-G-Py-Pu-3′ sequence, a break at the Py usually results in a secondary break at the base directly opposite to the Pu, leaving a single-base 5′-extension. For primary cleavage sites containing a 5′-G-Py-Py-3′ sequence, a break at the Py usually results in a secondary break at the base directly opposite to the Py, creating a blunt end. As proposed by Povirk et al., this model posits that after the primary cleavage event by bleomycin, the resulting Fe(III)•BLM product and peroxyl radical regenerates activated Fe•BLM in order to perform a secondary cleavage on the opposite DNA strand through a reorganization of DNA binding without dissociation of the BLM from the DNA (Figure 2.1).

Figure 2.1. Double-Strand DNA Cleavage Patterns Exibited by BLM.
In the Hecht laboratory, a model of BLM mediated double-stranded DNA cleavage was developed, based on the structural determination of Zn(II)•BLM A$_2$ bound to the self-complementary Dickerson-Drew dodecamer, by 2D NMR and molecular modeling (Figure 2.2). The findings were further reinforced by biochemical studies involving Fe(II)•BLM A$_2$. According to this model, BLM mediates double-strand cleavage of this DNA without structural reorganization. Fe(II)•BLM A$_2$ mediated cleavage of the Dickerson–Drew dodecamer results in the appearance of two major cleavage sites, 5'−GA$_5$−3' on one DNA strand and 5'−GC$_{11}$−3' on the other DNA strand. In the $^1$H NMR spectrum, intermolecular NOE contacts between the Dickerson-Drew dodecamer and the metal binding domain of Zn(II)•BLM A$_2$ placed the C4' hydrogens of A$_5$ and C$_{11}$ oriented towards each other in the minor groove as well as at relatively equal distances from the metal center of the Zn(II)•BLM A$_2$ (Figure 2.2). While structural variations between Zn(II)•BLM and Fe(II)•BLM must be taken into account, the correlation of NMR data and cleavage site analysis strongly support a model of BLM mediated double-strand DNA cleavage occurring without reorganization of the BLM on the DNA oligonucleotide.

**Figure 2.2.** NMR Model of DNA-Bleomycin Interaction Revealing the Potential Site of a Double-Strand Break.\textsuperscript{52}
Based on the previous findings in Hecht laboratory, the study of dsDNA cleavage was extended to a library of hairpin DNAs (Table 2.1)\textsuperscript{53}, selected for having high binding affinity to bleomycin As. All of the hairpin DNAs undergo multiple double-strand DNA cleavage events. Interestingly some of these cleavages involve a novel mechanism, not described before.

**Table 2.1.** Sequence of 10 Hairpin DNAs.\textsuperscript{53}

<table>
<thead>
<tr>
<th>DNA 1</th>
<th>DNA 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' AGATCATG</td>
<td>5' CTACCTAA</td>
</tr>
<tr>
<td>3' TCTAGTAC</td>
<td>3' GATGATTT</td>
</tr>
<tr>
<td>DNA 2</td>
<td>DNA 7</td>
</tr>
<tr>
<td>5' CGTGACGC</td>
<td>5' TACGGCA</td>
</tr>
<tr>
<td>3' GCACTGGG</td>
<td>3' ATGGCGGT</td>
</tr>
<tr>
<td>DNA 3</td>
<td>DNA 8</td>
</tr>
<tr>
<td>5' TAACTGGG</td>
<td>5' GGTACCT</td>
</tr>
<tr>
<td>3' ATCTACCC</td>
<td>3' GGCATGGA</td>
</tr>
<tr>
<td>DNA 4</td>
<td>DNA 9</td>
</tr>
<tr>
<td>5' GAGGAGAT</td>
<td>5' CGTGGGTA</td>
</tr>
<tr>
<td>3' CTCTCCTA</td>
<td>3' GCAACAAT</td>
</tr>
<tr>
<td>DNA 5</td>
<td>DNA 10</td>
</tr>
<tr>
<td>5' ACAAGAATA</td>
<td>5' CAGCCATTG</td>
</tr>
<tr>
<td>3' TGCTCTAT</td>
<td>3' GGSCTAAG</td>
</tr>
</tbody>
</table>

### 2.2. Results

In a previous report we showed the bleomycin A\textsubscript{5} cleavage sites on the 10 hairpin DNAs (Figure 2.3).\textsuperscript{54} To extend our knowledge further to bleomycin-induced double-strand cleavages, the hairpin DNA library seemed to be the ideal model. Each of these DNAs were alternatively labeled at 5'- and 3'-ends using \textsuperscript{32}P-phosphate and subjected to 5 \textmu M Fe(II)•bleomycin treatment for 30 minutes, following the method described in section 2.4. The double-strand cleavage products appeared as co-migrating bands when
analyzed on a native gel. When subsequently analyzed on a denaturing gel, the sites of cleavage could be determined at single nucleotide resolution.

Figure 2.3. Sites of BLM-Mediated Damage for the Ten Hairpin DNAs Studied.\textsuperscript{54}

The chemistry of coupled double-strand cleavage has been studied over a number of years\textsuperscript{16,17,55} and is illustrated in Scheme 2.1. The scheme illustrates an outline of the products formed at every step of bleomycin-induced DNA cleavage resulting in double-strand lesions. As evident in Scheme 2.1, the initial BLM induced lesion can produce an alkali labile lesion (product I), or to progress the strand scission process leading to a
nicked DNA duplex (product II). The nicked DNA can undergo a second Fe(II)•BLM mediated oxidative cleavage on the opposing strand of the DNA duplex, in close proximity to the first lesion (product IV), or to an alkali labile lesion (product III), depending on the availability of oxygen. Although product IV results in double-strand cleavage, treatment of III with $n$-butylamine would also lead to double-strand cleavage (product V).

**Scheme 2.1.** Mechanisms of Fe•Bleomycin Induced Double-Strand DNA Cleavage.\textsuperscript{56}
DNA 8, having multiple putative dsDNA cleavage sites, was chosen for the initial experiment. As shown in Figure 2.4, treatment with Fe•bleomycin A₅ formed three double-strand DNA cleavage products (b, c and d in lanes 2 and 4). It was also observed that the amounts of all of those cleavage products were enhanced following additional treatment with n-butylamine (products b, c and d in lanes 3 and 5), while the bands representing the full-length hairpin DNAs (a in lanes 2 and 4) were reduced in intensity. As illustrated in Figure 2.4 the species which co-migrate with full-length hairpin DNA (a in lanes 2, 3, 4 and 5) are products of type I, II and III (Scheme 2.1), with residual amounts of the unreacted full-length hairpin DNA.

**Figure 2.4.** Double-Strand Cleavage of [5'-³²P]-End Labeled (lanes 1 – 3) and [3'-³²P]-End Labeled (lanes 4 – 6) 64-Nucleotide Hairpin DNA 8 by Fe(II)•BLM A₅. Lane 1, [5'-³²P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A₅; lane 3, 5 µM Fe(II)•BLM A₅ + 2 mM n-butylamine; lane 4, 5 µM Fe(II)•BLM A₅; lane 5, 5 µM Fe(II)•BLM A₅ + 2 mM n-butylamine; lane 6, [3'-³²P]-end labeled DNA alone.
The positions of dsDNA cleavage in Figure 2.4 (bands b, c and d) were assigned by isolating each of the bands from the native gel and analyzing them in a 20% denaturing polyacrylamide gel in direct comparison with a Maxam–Gilbert G+A sequencing lane (Figure 2.5). As shown in Figure 2.5, the [3′-32P]-end labeled band 4b (Figure 2.4) co-migrated with the band in the sequencing lane corresponding to cleavage at T\(_{50}\) (Figure 2.5). Similarly, the product treated with \(n\)-butylamine (5b) was also cleaved at T\(_{50}\). Likewise, the other two bands (4c and 4d) were found to be cleaved at positions C\(_{52}\) and T\(_{56}\). As shown in lane 2 of Figure 2.5, product 4a produced bands which co-migrated with the products derived from 4b, 4c and 4d, indicating that the duplex in 4a only contained products with strand scission at those positions (T\(_{50}\), C\(_{52}\) and T\(_{56}\)) without any complementary cleavages on the opposite strand, suggesting that three sites represent primary sites of cleavage.

Following a similar process, [5′-32P]-end labeled products derived from hairpin DNA 8 were identified. As shown in Figure 2.5 (lanes 9, 11 and 12), the products in bands 2b, 2c and 2d (Figure 2.4) were cleaved at T\(_{14}\), G\(_{12}\) and A\(_9\), respectively. This suggests that the double-strand cleavage product represented in band b (Figure 2.4) must have resulted from cleavage at T\(_{14}\) and T\(_{50}\). Similarly, the product in band c resulted from cleavage at G\(_{12}\) and C\(_{52}\), whereas cleavage at A\(_9\) and T\(_{56}\) resulted in band d. The sequences of the double-strand cleavage products in DNA 8 and all the other members of the library are summarized in Figure 2.6. Additionally, as shown in lane 8 of Figure 2.5, band 2a (from Figure 2.4) co-migrated with bands corresponding to A\(_9\), G\(_{12}\) and T\(_{14}\) (Figure 2.5), suggesting that each of these positions can also be primary sites of cleavage.
Thus analysis of the dsDNA cleavage of DNA 8 indicates the absence of any obligatory order of cleavage seen in previous studies.

**Figure 2.5** Sequencing Gel Analysis of Fe(II)•BLM A5-Induced Double-Strand Cleavage Sites of [3'-32P]-End Labeled (lanes 1-6) and [5'-32P]-End Labeled (lanes 7-12) DNA 8. Each lane (except lanes 1 and 7) corresponds to a numbered cleavage band, shown in Fig. 2.4 Lane 1, Maxam–Gilbert G+A sequencing lane of [3'-32P]-end labeled DNA 8; lane 2, band 4a; lane 3, band 4b; lane 4, band 5b; lane 5, band 4c; lane 6, band 4d; lane 7, Maxam–Gilbert G+A sequencing lane of [5'-32P]-end labeled DNA 8; lane 8, band 2a; lane 9, band 2b; lane 10, band 3b; lane 11, band 2c; lane 12, band 2d.56
Figure 2.6. Summary of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNAs 1-10. Orange bases indicate the randomized region of the original hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to coupled double-strand cleavage events whereas red arrows indicate non-coupled double-strand cleavage events, which results from two independent single-strand cleavages on opposite strands. Nucleotides colored in red indicate primary sites of double-strand cleavage.56
As shown in Figure 2.7, five new bands appeared when hairpin DNA 2 was treated with Fe(II)•BLM A₅. Each of the five bands in lanes 2 and 3 along with the bands (2a and 3a) co-migrating with unreacted DNA were isolated and subjected to analysis by denaturing polyacrylamide gel electrophoresis.

**Figure 2.7.** Double-Strand Cleavage of [3′-³²P]-End Labeled (lane 2) and [5′-³²P]-End Labeled (lane 3) 64-Nucleotide Hairpin DNA 2 by Fe•BLM A₅. Lane 1, [3′-³²P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A₅; lane 3, 5 µM Fe(II)•BLM A₅; lane 4, [5′-³²P]-end labeled DNA alone.⁵⁶

As shown in Figure 2.8, the [5′-³²P]-end labeled DNA 2, when treated with bleomycin, was cleaved at positions A₉, T₁₃, A₁₅, C₁₆ and C₁₈, whereas the [3′-³²P]-end labeled bleomycin treated DNA 2 was cleaved at positions T₄₆, C₄₈, T₅₀, C₅₁ and T₅₆. Co-migrating bands suggests that DNA 2 involved dsDNA cleavage at T₄₆/C₁₈, C₄₈/C₁₆, T₅₀/A₁₅, C₅₁/T₁₃ and T₅₆/A₉. The sequences of dsDNA cleavage products are summarized in Figure 2.6.
Figure 2.8. Sequencing Gel Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites of [3'-32P]-End Labeled (lanes 1-7) and [5'-32P]-End Labeled (lanes 8-14) Hairpin DNA 2. Each lane (except lanes 1 and 8) corresponds to a numbered cleavage band, shown in Figure 2.7. Lane 1, Maxam–Gilbert G+A sequencing lane of [3'-32P]-end labeled DNA 2; lane 2, band 2f; lane 3, band 2e; lane 4, band 2d; lane 5, band 2c; lane 6, band 2b; lane 7, band 2a; Lane 8, Maxam–Gilbert G+A sequencing lane of [5'-32P]-end labeled DNA 2; lane 9, band 3f; lane 10, band 3e; lane 11, band 3d; lane 12, band 3c; lane 13, band 3b; lane 14, band 3a. 

Additionally, analysis of 3a (Figure 2.7) by denaturing polyacrylamide gel electrophoresis (Figure 2.8) indicated cleavage at positions C\textsubscript{48} and T\textsubscript{50}. In comparison, analysis of band 4a (Figure 2.7) indicated cleavage at positions T\textsubscript{13} and C\textsubscript{18} (Figure 2.8).

As noted in Scheme 2.1, these bands must represent sites nicked to produce intermediates.
of type II or III (Scheme 2.1), without associated cleavage on the opposite strand, thus indicating primary sites of cleavages. Similarly, bands 2f/3f, when compared in a denaturing gel (Figure 2.8), showed little partially cleaved material corresponding to products derived from bands 2a/3a suggesting that the cleavages at A$_9$ and T$_{56}$ represent independent single strand cleavage events, resulting in adventitious dsDNA cleavage product. In comparison, the rest of the cleavage sites (T$_{13}$/C$_{51}$, A$_{15}$/T$_{50}$, C$_{16}$/C$_{48}$ and C$_{18}$/C$_{46}$) appeared to be coupled dsDNA cleavages with obligatory primary sites, consistent with the mechanism described previously.$^{57}$

The remaining eight hairpin DNAs in a library were also analyzed in a similar fashion to establish patterns of double-strand cleavage by Fe(II)$\cdot$BLM A$_5$. Among the original 14 cleavage sites in hairpin DNA 1 (Figure 2.3),$^{54}$ six sites were located within the original eight base-pair randomized region that had been used to create the random DNA library.$^{57}$ Interestingly, native gel analysis revealed only one double-strand cleavage product (Figure 2.9A). Further analysis of the cleavage products (Figure 2.9B) revealed that bands 2b and 3b in Figure 2.9A, contained a mixture of two sets of products, resulting from cleavages at A$_9$/T$_{56}$ and T$_{10}$/A$_{55}$ neither of which resulted from coupled double-strand cleavage.
Figure 2.9. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 1. (A) Double-strand cleavage of [3'-32P]-end labeled (lanes 1 and 2) and [5'-32P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 1 by bleomycin A5. Lane 1, [3'-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A5; lane 3, 5 µM Fe(II)•BLM A5; lane 4, [5'-32P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3'-32P]-end labeled (lanes 1-3) and [5'-32P]-end labeled (lanes 4-6) DNA 1. Each lane (except lanes 3 and 4) corresponds to a numbered cleavage band, shown in (A). Lane 1, band 2b; lane 2, band 2a; lane 3, Maxam–Gilbert G+A sequencing lane of [3'-32P]-end labeled DNA 1; lane 4, Maxam–Gilbert G+A sequencing lane of [5'-32P]-end labeled DNA 1; lane 5, band 3b; lane 6, band 3a.56
Hairpin DNA 3, shown in Figure 2.6, displayed two pairs of co-migrating bands in the native polyacrylamide gel analysis (Figure 2.10A). Further analysis of those bands by denaturing polyacrylamide gel electrophoresis (Figure 2.10B) indicated that they are the products from coupled double-strand cleavage at T₁₅/C₄₉, having T₁₅ as the primary site of cleavage and non-coupled double-strand cleavage involving T₁₀ and A₅₅.

**Figure 2.10.** Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 3. (A) Double strand cleavage of [3′-³²P]-end labeled (lanes 1 and 2) and [5′-³²P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 3 by bleomycin A₅. Lane 1, [3′-³²P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A₅; lane 3, 5 µM Fe(II)•BLM A₅; lane 4, [5′-³²P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3′-³²P]-end labeled (lanes 1-4) and
[5′-32P]-end labeled (lanes 5-8) DNA 3. Each lane (except lanes 1 and 5) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam–Gilbert G+A sequencing lane of [3′-32P]-end labeled DNA 3; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; Lane 5, Maxam–Gilbert G+A sequencing lane of [5′-32P]-end labeled DNA 3; lane 6, band 3a; lane 7, band 3b; lane 8, band 3c.56

Similarly, hairpin DNA 4 showed two double-strand cleavage bands (Figure 2.11A). Each of the bands when, analyzed on a denaturing polyacrylamide gel (Figure 2.11B), revealed dsDNA cleavage at A9/T56 and T10/A55. Analysis of bands 2a and 3a containing unreacted products suggested that neither was involved in a coupled double-strand cleavage event.

**Figure 2.11.** Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 4. (A) Double strand cleavage of [3′-32P]-end labeled (lanes 1 and 2) and
Hairpin DNA 5 exhibited three sets of double-strand cleavages (Figure 2.12A), although a total of 27 sites of bleomycin A₅ mediated cleavages had been reported earlier (Figure 2.3).²⁴ Each of these bands, on a denaturing polyacrylamide gel (Figure 2.12B), indicated a single duplex resulting from double-strand cleavage at C₁₂/T₅₄, T₁₀/T₅₆ and A₁₅/T₄₉. Only the site involving C₁₂/T₅₄ represented a coupled double strand cleavage event, with T₅₄ being the primary cleavage site.
Figure 2.12. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 5. (A) Double strand cleavage of [3′-32P]-end labeled (lanes 1 and 2) and [5′-32P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 5 by bleomycin A5. Lane 1, [3′-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A5; lane 3, 5 µM Fe(II)•BLM A5; lane 4, [5′-32P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3′-32P]-end labeled (lanes 1-5) and [5′-32P]-end labeled (lanes 6-10) DNA 5. Each lane (except lanes 5 and 10) corresponds to a numbered cleavage band, shown in (A). Lane 1, band 2d; lane 2, band 2c; lane 3, band 2b; lane 4, band 2a; lane 5, Maxam–Gilbert G+A sequencing lane of [3′-32P]-end labeled DNA 5; lane 6, band 3d; lane 7, band 3c; lane 8, band 3b; lane 9, band 3a; Lane 10, Maxam–Gilbert G+A sequencing lane of [5′-32P]-end labeled DNA 5.56

The study of hairpin DNA 6, as illustrated in Figure 2.13A, revealed two putative double-strand cleavage products. Analysis of those bands revealed two sets of dsDNA cleavage products one of which involves T12 and T32, with T32 being the primary site of
cleavage. The second dsDNA cleavage product at A₉ and T₅₆, resulted from non-coupled dsDNA cleavage with no obligatory primary site of cleavage.

Figure 2.13. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 6. (A) Double strand cleavage of [3′⁻³²P]-end labeled (lanes 1 and 2) and [5′⁻³²P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 6 by bleomycin A₅. Lane 1, [3′⁻³²P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A₅; lane 3, 5 µM Fe(II)•BLM A₅; lane 4, [5′⁻³²P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3′⁻³²P]-end labeled (lanes 1-4) and [5′⁻³²P]-end labeled (lanes 5-8) DNA 6. Each lane (except lanes 1 and 5) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam–Gilbert G+A sequencing lane of [3′⁻³²P]-end labeled DNA 6; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; lane 5, Maxam–Gilbert G+A sequencing lane of [5′⁻³²P]-end labeled DNA 6; lane 6, band 3a; lane 7, band 3b; lane 8, band 3c.⁵⁶
The five double-strand cleavage products from DNA 7 (Figure 2.14) observed on a native polyacrylamide gel (Figure 2.14A), were isolated and analyzed on a denaturing polyacrylamide gel (Figure 2.14B). Three duplex products resulted from

**Figure 2.14.** Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 7. (A) Double-strand cleavage of [3'-32P]-end labeled (lanes 1 and 2) and [5'-32P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 7 by bleomycin A5. Lane 1, [3'-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A5; lane 3, 5 µM Fe(II)•BLM A5; lane 4, [5'-32P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3'-32P]-end labeled (lanes 1-7) and [5'-32P]-end labeled (lanes 8-14) DNA 7. Each lane (except lanes 1 and 14) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam–Gilbert G+A sequencing lane of [3'-32P]-end labeled DNA 7; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; lane 5, band 2d; lane 6, band 2e; lane 7, band 2f; lane 8, band 3f; lane 9, band 3e; lane 10, band 3d; lane 11, band 3c; lane 12, band 3b; lane 13, band 3a; lane 14, Maxam–Gilbert G+A sequencing lane of [5'-32P]-end labeled DNA 7.56
coupled double-strand cleavage events. These included sites involving T_{11}/T_{53}, C_{13}/C_{51} and C_{17}/T_{47}. In these, T_{53}, C_{51} and C_{17} were the primary sites of cleavages. The other double-strand cleavage products involving cleavage at T_{10}/A_{55} and C_{15}/C_{49} represented non-coupled dsDNA cleavage events.

When DNA 9 (Figure 2.15) was analyzed for dsDNA cleavage as shown in Figure 2.15A, three double-strand cleavage products were observed. The denaturing polyacrylamide gel (Figure 2.15B) of those bands indicated that the sites involved cleavage at T_{10}/A_{55}, T_{13}/A_{52} and T_{16}/A_{49}. Analysis of the mixed products 2a and 3a (Figure 2.15A) revealed four primary sites of cleavages at T_{10}, T_{13}, T_{16} and A_{55} (Figure 2.15B), suggesting that cleavage at T_{13}/A_{52} and T_{16}/A_{49} represent coupled dsDNA cleavage events whereas that at T_{10}/A_{55} indicates a non-coupled event.
Figure 2.15. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 9. (A) Double-strand cleavage of [3′-32P]-end labeled (lane 2) and [5′-32P]-end labeled (lane 3) 64-nucleotide hairpin DNA 9 by Fe•bleomycin A5. Lane 1, [3′-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A5; lane 3, 5 µM Fe(II)•BLM A5; lane 4, [5′-32P]-end labeled DNA alone. (B) Sequencing gel analysis of Fe•bleomycin-induced double-strand cleavage sites of [3′-32P]-end labeled (lanes 1-5) and [5′-32P]-end labeled (lanes 6-10) hairpin DNA 9. Each lane (except lanes 5 and 10) corresponds to a numbered cleavage band, shown in (A). Lane 1, band 2d; lane 2, band 2c; lane 3, band 2b; lane 4, band 2a; lane 5, Maxam–Gilbert G+A sequencing lane of [3′-32P]-end labeled DNA 9; lane 6, band 3d; lane 7, band 3c; lane 8, band 3b; lane 9, band 3a; Lane 10, Maxam–Gilbert G+A sequencing lane of [5′-32P]-end labeled DNA 9.56

The last member of the library, DNA 10, showed three double-strand cleavage products after Fe•BLM treatment as shown in Figure 2.16A. The co-migrating bands, when analyzed on a denaturing polyacrylamide gel (Figure 2.16B), indicated two separate coupled, double-strand cleavages. The cleavage sites involved C_{11}/C_{53}, with the
latter being the primary site of cleavage and at C<sub>13</sub>/G<sub>52</sub>, with the former being the primary site. The third product, involving T<sub>10</sub>/A<sub>55</sub> was a non-coupled cleavage event with no preferred primary cleavage site.

Figure 2.16. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 10. (A) Double-strand cleavage of [3′-32P]-end labeled (lanes 1 and 2) and [5′-32P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 10 by bleomycin A₅. Lane 1, [3′-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A₅; lane 3, 5 µM Fe(II)•BLM A₅; lane 4, [5′-32P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3′-32P]-end labeled (lanes 1-5) and [5′-32P]-end labeled (lanes 6-10) DNA 10. Each lane (except lanes 1 and 6) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam–Gilbert G+A sequencing lane of [3′-32P]-end labeled DNA 10; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; lane 5, band 2d; lane 6, Maxam–Gilbert G+A sequencing lane of [5′-32P]-end labeled DNA 10; lane 7, band 3a; lane 8, band 3b; lane 9, band 3c; Lane 10, band 3d.⁵⁶
2.3. Discussion

Although bleomycin has been used as an antitumor agent for decades, the mechanism by which it exerts its therapeutic effect has never been fully established. Additionally, the very small clinical dose also suggests the presence of multiple highly specific mechanisms to achieve tumor cell killing. The recent use of a hairpin DNA library, selected for tight binding to the drug, in bleomycin cleavage studies has confirmed our early findings that model hairpin DNAs having a single strong bleomycin binding site can be cleaved in multiple places.\textsuperscript{58} Additionally, we found that those cleavages include numerous double-strand cleavages, which may suggest a mechanism for tumor cell killing.

Bleomycin induced double-strand cleavage of DNA was first studied extensively by the Povirk laboratory.\textsuperscript{51,59-61} In their study, three linear DNA duplexes containing 250 base pairs were analyzed for dsDNA cleavages, which showed 26 double-strand DNA cleavage sites. The sites of double-strand cleavage, resulting from treatment with Fe(II)•BLM A\textsubscript{2}, were analyzed by separation of the DNA fragments on a native polyacrylamide gel. An analysis of each of the bands following 5′ or 3′-\textsuperscript{32}P-end labeling was carried out using a sequencing gel. With one exception, all of the double-strand breaks showed a G-Py sequence on one strand and a much greater diversity of cleavage products on the other strand. Interestingly, eleven of the dsDNA cleavages produced blunt-end products, whereas twelve of them resulted in products with one nucleotide 5′-extensions.\textsuperscript{51}

As they had maintained the condition of single hit kinetics throughout their studies, Povirk et al.\textsuperscript{51} concluded that each dsDNA cleavage observed in their analysis
must have resulted from the action of a single bleomycin molecule. The appearance of a G-Py sequence virtually in all of the dsDNA cleavages suggested that this must be the primary site of cleavage. The occasional formation of alkali labile lesions was also observed by the researchers at the secondary sites. In a different study by Keller and Oppenheimer, an enhanced cleavage opposite to an initial bleomycin-induced nick had also been reported. Absalon et al. explained the nature of dsDNA cleavages in a more detailed way in a subsequent study, utilizing hairpin DNA oligonucleotides. Employing the technique of labeling their hairpin DNAs internally at a \(^{32}\)P-labeled phosphate, they were able to determine the ratio of single-strand and double-strand cleavage at each individual site. Replacing the C-4' H atom, susceptible to abstraction by Fe•BLM by deuterium in putative bleomycin cleavage site, they concluded that bleomycin induced dsDNA cleavage results from a single bleomycin molecule and requires reactivation of bleomycin after the first cleavage event (Figure 2.17).

In a previous study we reported a hairpin DNA with one bleomycin cleavage site that undergoes highly efficient cleavage at that site. The method involved alternatively labeling the hairpin DNAs at 5'- and 3'-ends with a radiolabeled \(^{32}\)P-ATP. For coupled dsDNA cleavage events (Figure 2.17) the ratio of cleavage products observed for the two labeled hairpin DNAs varied dramatically, which enabled quantification of frank-strand scission and alkali labile lesion.

In our present study, the alternatively labeled 5' and 3'-end cleavage products also produced bands of different intensities on the native gel, suggesting that some of those cleavages involve dsDNA cleavages. However, presence of a large number of dsDNA cleavage bands in the hairpin DNAs prompted us to employ a modified technique similar
to one described by Povirk et al.\textsuperscript{51} In addition to studying the bands containing dsDNA cleavage products, we also analyzed the bands at the top of each cleavage lanes on a non-denaturing polyacrylamide gel. As described in Scheme 2.1, these bands contained a mixture of products with unreacted hairpin DNA. Among all the different types of products in the mixture, product III (Scheme 2.1) had a frank strand scission on a primary site of bleomycin cleavage and an alkali labile lesion on the other strand, affording a hairpin DNA nicked at the primary site. As suggested by Povirk et al.,\textsuperscript{51} product III resulted from reactivation of a bleomycin molecule after the frank strand scission at the primary site, leading to an alkali labile lesion on the second strand instead of a second strand break. Product III also carries the information of a primary bleomycin induced dsDNA cleavage site when analyzed on a denaturing polyacrylamide gel. As illustrated in Figure 2.3, treatments of bands 2a and 3a with \textit{n}-butylamine resulted in additional dsDNA cleavage products in lanes 3 and 5, which suggested the presence of product III.

Additionally, coupled double-strand bleomycin cleavage events were identified by analyzing the top bands from each native gel. As shown in Figure 2.15, the DNA cleavage product III gave a band at the primary cleavage site on a denaturing polyacrylamide gel, which came from the singly nicked DNA. The strand containing the alkali labile lesion failed to produce a similar band on a denaturing gel, confirming that it was a secondary site of cleavage. These phenomena were further illustrated in Figure 2.15B. 3′-\textsuperscript{32}P-end labeled DNAs recovered from bands 2d, 2c and 2b (Figure 2.15A), respectively, revealed the sites of bleomycin induced dsDNA cleavage at the 3′ labeled side of the hairpin DNAs at A\textsubscript{55}, A\textsubscript{52} and A\textsubscript{49}, respectively (lanes 1, 2 and 3 in Figure
Lane 4 of Figure 2.15 B, containing band 2a from Figure 2.15A, showed a strong cleavage at A55, but weak cleavages at A52 and A49. Similarly, lanes 6, 7 and 8 of Figure 2.17.

**Figure 2.17.** Bleomycin Induced Double-Strand DNA Lesion Progresses Through Either (A) A Coupled Cleavage Event or (B) A Non-Coupled Cleavage Event. The coupled DNA cleavage is mediated by a single BLM molecule, which is reactivated after the primary lesion to produce a secondary cleavage on the opposing strand of DNA, without dissociating from the site of cleavage. The non-coupled cleavage is mediated by two BLM molecules, and involves independent activation and association of BLM molecules with cleavage sites, closely situated on the opposing strands of DNA.

Figure 2.15B contained 5′-32P-end labeled cleavage products recovered from bands 3d, 3c and 3b, respectively (Figure 2.15A). Those bands readily revealed the sites of bleomycin induced dsDNA cleavage on the 5′ side of the hairpin DNA at T10, T13 and T16, respectively. Lane 9 (Figure 2.15B), containing product 3a (Figure 2.15A), produced strong bands at sites T10, T13 and T16. The absence of intense bands at A52 and A49 at the BLM-treated 3′-32P-end labeled side of the hairpin DNA suggests that these sites contained alkali labile lesions (lane 4, Figure 2.15A). Additionally it proves that cleavages at T13/A52 and T16/A49 are coupled with T13 and T16 as the primary sites of cleavage. The appearance of both the cleavage partners of the A55/T10 in lanes 4 and 9,
respectively suggests that either of these sites can act as a primary site of cleavage in a dsDNA cleavage event or they result from two independent single strand cleavages induced by two separate bleomycin molecules, resulting in an adventitious dsDNA cleavage. Although the reactions were not carried out under single-hit kinetic condition to assure whether the cleavages at T_{10}/A_{55} involved a single bleomycin molecule, the earlier observations made by Povirk suggest that the cleavage at T_{10}/A_{55} represented two independent events.

In the study of dsDNA cleavage done by Povirk et al., all but one of the dsDNA cleavage sites involved a G-Py sequence as one of the cleavage sites and the opposing strand mostly involved a non-conventional site, not usually seen as a single-strand cleavage site. In our present study all 14 confirmed coupled double-strand cleavage events involved a G-Py sequence as a primary site. In the previous study, seven of the cleavages had a G-Py-Py-Pu sequence as the primary site with the initial cleavage at the first Py residue following the guanosine. The subsequent cleavage on the opposing strand produced blunt ended products. Six of the other cleavages involved G-Py-Py-Pu sequences and the cleavage at the first Py resulted in either blunt-end products or products with a single-base 5’ overhang on the opposing strand. In our study, four of the cleavage products involved G-Py-Py-Pu sequences and none had G-Py-Py-Py sequences (Table 2.2). In the earlier study, six of the dsDNA cleavages were also found at G-Py-Pu-Py sequences, whereas three at G-Py-Pu-Pu sequences. In all of those cleavages, the strand scission resulted in products with one-base 5’ overhangs. In the current study, three of the double-strand events involved G-Py-Pu-Py sequences and, more interestingly, seven of the 14 cleavages involved G-Py-Pu-Pu sequences (Table 2.2). Overall, our
recent study produced similar results as those reported by Povirk et al., although the frequency of double-strand cleavages on DNAs strongly bound by bleomycin was somewhat greater than reported earlier.

**Table 2.2.** Coupled Double-Strand BLM Cleavage Sites in Hairpin DNAs 1 – 10

<table>
<thead>
<tr>
<th>DNA 1</th>
<th>G-Py-Py-Pu</th>
<th>G-Py-Py-Py</th>
<th>G-Py-Py-Py</th>
<th>G-Py-Pu-Pu</th>
</tr>
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<tbody>
<tr>
<td>3'</td>
<td>ATCATGCTAGA</td>
<td>ACTGCTAGA</td>
<td>ACTGCTAGA</td>
<td>ACTGCTAGA</td>
</tr>
<tr>
<td>5'</td>
<td>ATCATGCTAGA</td>
<td>ACTGCTAGA</td>
<td>ACTGCTAGA</td>
<td>ACTGCTAGA</td>
</tr>
</tbody>
</table>

**DNA 2**

| 3' | GGCTGCTAGA | GCTGCTAGA | GCTGCTAGA | GCTGCTAGA |
| 5' | GGCTGCTAGA | GCTGCTAGA | GCTGCTAGA | GCTGCTAGA |

**DNA 3**

| 3' | GTGGCACC | GTGGCACC | GTGGCACC | GTGGCACC |
| 5' | GTGGCACC | GTGGCACC | GTGGCACC | GTGGCACC |

**DNA 4**

| 3' | CTCTCCTTCTTTT | CTCTCCTTCTTTT | CTCTCCTTCTTTT | CTCTCCTTCTTTT |
| 5' | CTCTCCTTCTTTT | CTCTCCTTCTTTT | CTCTCCTTCTTTT | CTCTCCTTCTTTT |

**DNA 5**

| 3' | GTTAGTCATA | GTTAGTCATA | GTTAGTCATA | GTTAGTCATA |
| 5' | GTTAGTCATA | GTTAGTCATA | GTTAGTCATA | GTTAGTCATA |

**DNA 6**

| 3' | GTAGCATTC | GTAGCATTC | GTAGCATTC | GTAGCATTC |
| 5' | GTAGCATTC | GTAGCATTC | GTAGCATTC | GTAGCATTC |

**DNA 7**

| 3' | GCCGTTGAA | GCCGTTGAA | GCCGTTGAA | GCCGTTGAA |
| 5' | GCCGTTGAA | GCCGTTGAA | GCCGTTGAA | GCCGTTGAA |

**DNA 8**

| 3' | GGCCTAGCTTAA | GGCCTAGCTTAA | GGCCTAGCTTAA | GGCCTAGCTTAA |
| 5' | GGCCTAGCTTAA | GGCCTAGCTTAA | GGCCTAGCTTAA | GGCCTAGCTTAA |

**DNA 9**

| 3' | GTTGTTTAAA | GTTGTTTAAA | GTTGTTTAAA | GTTGTTTAAA |
| 5' | GTTGTTTAAA | GTTGTTTAAA | GTTGTTTAAA | GTTGTTTAAA |

**DNA 10**

| 3' | GCCACCATTTT | GCCACCATTTT | GCCACCATTTT | GCCACCATTTT |
| 5' | GCCACCATTTT | GCCACCATTTT | GCCACCATTTT | GCCACCATTTT |

aCleavage sequences aligned with primary G-Py cleavage sites.
Besides the coupled double-strand cleavages summarized in Table 2.1, the other 17 non-coupled dsDNA cleavages (Figure 2.17B) illustrated in Table 2.3 are

**Table 2.3. Non-Coupled Double-Strand BLM Cleavage Sites in Hairpin DNAs 1 – 10**

<table>
<thead>
<tr>
<th>Hairpin DNA</th>
<th>Sequences cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA 1</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGATCATCAGA</td>
<td>AATA ATAG</td>
</tr>
<tr>
<td>3′ TTAATCTAGACTT</td>
<td>TTA TAT</td>
</tr>
<tr>
<td><strong>DNA 2</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGCTGACGCAGA</td>
<td>AATC</td>
</tr>
<tr>
<td>3′ TTAATCCTGCCCTT</td>
<td>TTAG</td>
</tr>
<tr>
<td><strong>DNA 3</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATATTAGTGCAAA</td>
<td>AATA</td>
</tr>
<tr>
<td>3′ TTAATCTACCCCTT</td>
<td>TAAT</td>
</tr>
<tr>
<td><strong>DNA 4</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGAGAGGATAA</td>
<td>AATG ATGA GAAG</td>
</tr>
<tr>
<td>3′ TTAATCTCTCTATTT</td>
<td>TTAG TACT CTCT</td>
</tr>
<tr>
<td><strong>DNA 5</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATACAGAAATAAA</td>
<td>ATAC GAAT</td>
</tr>
<tr>
<td>3′ TTAATGTCTTTTATTT</td>
<td>TAGA CTTA</td>
</tr>
<tr>
<td><strong>DNA 6</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGCACCTAAA</td>
<td>AAAC</td>
</tr>
<tr>
<td>3′ TTAATGTGATTTTT</td>
<td>TTAG</td>
</tr>
<tr>
<td><strong>DNA 7</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATACGCGGCAGA</td>
<td>AATA GCAC</td>
</tr>
<tr>
<td>3′ TTAATGCGCGTTTT</td>
<td>TATT GCAC</td>
</tr>
<tr>
<td><strong>DNA 8</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGGTACCCTAA</td>
<td>AATG GGTC GTAC</td>
</tr>
<tr>
<td>3′ TTAATCCATCGGATT</td>
<td>TTAG CCCA CTGC</td>
</tr>
<tr>
<td><strong>DNA 9</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGTTGTTAAA</td>
<td>ATCG</td>
</tr>
<tr>
<td>3′ TTAATGCAACAATTT</td>
<td>TAGC</td>
</tr>
<tr>
<td><strong>DNA 10</strong></td>
<td></td>
</tr>
<tr>
<td>5′ AATGGCCATGGA</td>
<td>ATCG</td>
</tr>
<tr>
<td>3′ TTAATGCGCTACTT</td>
<td>TAGC</td>
</tr>
</tbody>
</table>

*All sequences aligned from 5′-end of hairpin DNAs.*

43
unprecedented. None of these cleavages was of a type previously observed by Povirk et al.\textsuperscript{51} in their early studies. Interestingly, no sequence preferences have been observed in this particular kind of cleavage other than an appearance at AT-rich sequences. Most of these cleavages occurred at the non-randomized region of the hairpin DNAs, and were concluded to be a result of experimental design. However, a few of those cleavages occurred in the randomized regions of the hairpin DNAs, such as GAGA in DNA 4, a GAAT in DNA 5 and a GCGC in DNA 7. The only exception was DNA 8, which had two of those sites in the eight base pair long variable region, identified as GTAC and GGGT.

In a recent report, we have used three of those DNAs in a study involving surface plasmon resonance to elucidate bleomycin binding sites on those DNAs.\textsuperscript{58} DNAs 2, 4 and 5 in that study revealed one strong bleomycin binding site on each of those DNAs and a few weak binding sites. In our current study, each of those DNAs was observed to undergo multiple cleavages in close proximity, including single and double strand cleavage. The competition experiment involving DNA 2 and a 16-nucleotide hairpin DNA with a single bleomycin cleavage site indicated that even though bleomycin dissociates from the cleavage site on DNA 2, detectable by surface plasmon resonance spectroscopy (SPR), it did not cleave the 16-nucleotide hairpin DNA when both were present at the same concentration.

The model derived from these observations suggests that the hairpin DNAs are closely associated with Fe•BLM, even when not bound to them as judged by SPR. This strong association of bleomycin with the hairpin DNAs apparently provides enough opportunity for multiple cleavages in close proximity to the site of interaction. In our
study, both DNA 2 and 7, being the tightest binders\textsuperscript{57} of bleomycin A\textsubscript{5} underwent five pairs of dsDNA cleavages whereas the DNAs 1, 3 and 6, being weak binders,\textsuperscript{57} produced two dsDNA cleavages. Chromosomal DNAs may have regions, which bind to Fe•BLM more tightly than any DNA used in our study. Assuming that the pattern of bleomycin-induced dsDNA cleavage seen in the present study is more general, those regions in the chromosome may undergo extensive dsDNA cleavage, leading to deletions in gene structure. These deletions being difficult to repair may potentially be the cause of cell death, which would explain the potency of Fe•bleomycin in tumor cell killing.

2.4. Experimental

2.4.1. Materials.

T4 polynucleotide kinase was purchased from New England Biolabs. Recombinant terminal deoxynucleotidyl transferase was obtained from Roche. [\gamma-\textsuperscript{32}P]-ATP and [\alpha-\textsuperscript{32}P]-cordycepin were purchased from Perkin Elmer. Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4})\textsubscript{2}•6H\textsubscript{2}O and Chelex 100 were from Sigma Aldrich. The hairpin DNAs were obtained from Integrated DNA Technologies, Inc.

2.4.2. End Labeling and Purification of Hairpin DNAs.

The hairpin DNAs were end labeled using [\gamma-\textsuperscript{32}P]ATP + T4 polynucleotide kinase and [\alpha-\textsuperscript{32}P]-cordycepin + terminal deoxytransferase at the 5’ and 3’ -ends, respectively. Ten pmol of 64-nucleotide hairpin DNAs were [5’-\textsuperscript{32}P]-end labeled by incubation with 20 units of T4 polynucleotide kinase and 0.06 mCi [\gamma-\textsuperscript{32}P]ATP (specific activity 6000 Ci (222 TBq)/mmol) in 50 μL (total volume) of 70 mM Tris-HCl buffer, pH 7.6, containing
10 mM MgCl₂ and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by DNA purification by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h. The [3′-³²P]-end labeling was done by incubating 10 pmol of hairpin DNA with 20 units of terminal deoxynucleotidyl transferase and 0.06 mCi [α-³²P]-cordycepin (specific activity 6000 Ci (222 TBq)/mmol) in 50 μL (total volume) of 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl₂, 10 mM CoCl₂ and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by purification of DNA by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h.

2.4.3. Double-Strand DNA Cleavage of Hairpin DNAs.

Bleomycin cleavage of [5′-³²P]- and [3′-³²P]-end labeled hairpin DNAs was performed by incubating the hairpin DNAs (~30000 cpm) with 5 μM Fe²⁺ and 5 μM bleomycin A₅ at 25 °C for 30 min in a solution containing 10 μL of 2 mM MgCl₂ and 10 mM Na cacodylate, pH 7.0. Two μL of native gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 40% D-sucrose were added to the reaction mixture, which was separated on a 20% native polyacrylamide gel (200 V, 16 h, 4 °C). Double-strand cleavage sites were identified by visualizing gels using a phosphorimager.

2.4.4. n-Butylamine Treatment of Hairpin DNA 8.

Bleomycin cleavage reactions of [5′-³²P]-end and [3′-³²P]-end labeled DNA 8 were further treated with 2 mM n-butylamine and incubated at 25 °C for 10 min. The supernatants were removed under diminished pressure and, DNA pellets were washed with 10 μL of deionized water. The final solutions were mixed with 2 μL of native gel
loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 40% D-sucrose and separated on 20% native polyacrylamide gels (200 V, 16 h, 4 °C).

2.4.5. Maxam-Gilbert Sequencing Reaction.\textsuperscript{65}

Ten µL of [5′-\textsuperscript{32}P]- and [3′-\textsuperscript{32}P]-end labeled DNAs (~50000 cpm) were treated with 25 µL of formic acid and incubated at 25 °C for 4-5 min. The reactions were stopped by treatment with 200 µL of 0.3 M NaOAc, pH 7.0, 0.1 mM EDTA, and 25 µg/mL tRNA. The resulting solutions were mixed with 700 µL of ethanol and the DNAs were precipitated. The DNA pellets were washed twice with 70% ethanol, and the pellets were resuspended in 75 µL of 10% piperidine. The reaction mixtures were incubated at 90 °C for 30 min, and the cooled supernatants were concentrated under diminished pressure. The DNA pellets were washed with small amounts of water to remove residual piperidine and mixed with denaturing loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue and 1% xylene cyanol. The combined solutions were heated at 90 °C for 10 min and used as the sequencing lanes to compare [5′-\textsuperscript{32}P]-end and [3′-\textsuperscript{32}P]-end labeled DNAs on denaturing polyacrylamide gels.

2.4.6. Denaturing Gel Electrophoresis of DNA Cleavage Products.

The [5′-\textsuperscript{32}P]-end and [3′-\textsuperscript{32}P]-end labeled double-strand DNA cleavage bands visualized by native gel electrophoresis were excised from the gels and purified by ethanol precipitation and then mixed with 5 µL of denaturing gel loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue and 1% xylene cyanol. Following heating at 90 °C for 10 min, five-µL aliquots of the final solutions were chilled
on ice and separated on a 16% denaturing polyacrylamide gels containing 16% urea, along with 2 μL of [5′-32P]- and [3′-32P]-end labeled Maxam-Gilbert sequencing lanes to determine the sequences of the cleavage sites. The gels were visualized using a phosphorimager.
CHAPTER 3
EFFECT OF ABERRANT CYTIDINE METHYLATION ON BLEOMYCIN
INTERACTION WITH DNA

3.1. Introduction

Cytidine methylation is a key factor in epigenetic gene regulation as well as cancer biogenesis. Characterized by its dynamic nature, the methylation pattern changes in cancer cells and is marked by global hypo-methylation, although localized CpG-cytidine hypermethylation has been reported in some cancers. Genomic cytidine hypomethylation, as related to cancer, holds the promise of additional information about the nature of interactions between DNA and DNA-binding therapeutic antitumor agents such as bleomycin. The role of bleomycin as a therapeutic agent, related to the treatment of cancer, has been discussed in Chapter 1. As illustrated in Chapter 2, the anti-tumor activity of bleomycin has been attributed to the sequence selective cleavage of DNAs and RNAs mediated by activated Fe•BLM. Although highly efficient for single strand cleavages, the cytotoxicity of bleomycin is largely derived from a more potent form of sequence specific double-strand DNA (dsDNA) cleavage.

Recent studies involving the use of hairpin DNAs strongly bound by bleomycin revealed that 10-20 percent of all bleomycin induced DNA cleavages can lead to double-strand cleavages, with an appearance at G-pyrimidine (GC or GT) sites in almost every reported case. As demonstrated in the literature, the reduction of bleomycin induced strand scission upon methylation of the substrate DNA provided a good starting point to understand the basis of the potency of bleomycin as a tumor cell killing
In the present study, we employed three of those strongly bound hairpin DNAs (Figure 3.1) and a concentration comparable to the clinical dose of bleomycin A₅ (Figure 3.2) to study the dynamics of the interaction of bleomycin with DNA. In addition, the methylated counterparts of the same DNAs were used to further study bleomycin mediated DNA binding and subsequent double-strand cleavage.

**Figure 3.1.** 64-Nucleotide DNAs, Their Methylated Counterparts and the 16-Nucleotide DNA-C₁₁₅. The red cytidine with double asterisks indicates pro-fluorescent C₁₁₅.⁷⁹
3.2. Results

3.2.1. Competition Assay and the Tight Binders of Fe•BLM.

A competition assay, based on the inhibition of bleomycin induced cleavage of a 16-nucleotide hairpin DNA containing a fluorescent nucleotide (Figure 3.1) at the site of cleavage, has been employed to measure the effect of cytidine methylation on binding with bleomycin quantitatively. The 16-nucleotide DNA is known to be an efficient substrate for bleomycin A₅. The intact 16-nucleotide DNA does not fluoresce upon excitation because of quenching by nucleotides within the hairpin DNA. However, when the DNA is cleaved by Fe•BLM, the fluorescent nucleobase is released and fluoresces upon being excited at 310 nm. Admixture of an equivalent of a strongly bound 64-nucleotide hairpin DNA to the reaction mixture inhibits binding to the 16-nucleotide DNA and results in a proportionate decrease in fluorescence, which directly reflects the binding affinity of the 64-nucleotide DNA to bleomycin.
The main focus of this study was 64-nucleotide hairpin DNAs such as DNA 7, DNA 10 and DNA 11 (Figure 3.1), which have strong affinity for bleomycin, both in terms of binding (DNA 7 in Figure 3.3) and subsequent cleavage (DNA 7 in Figure 3.4). As shown in Figure 3.3, the gradual increase of the concentration of DNA 7 in the subsequent competition experiments involving 1 µM 16-nucleotide hairpin DNA significantly decreased the fluorescent peak of the 16-nucleotide hairpin DNA at 455 nm. At 1 µM concentration, the fluorescent peak was reduced to ~25% of the control peak.

**Figure 3.3.** Fluorescence Emission Spectra Resulting from Treatment of 16-nt Hairpin DNA-C<sub>f15</sub> With Fe(II)•BLM in the Presence or Absence of 64-nt Hairpin DNA 7. Reaction mixture contained 1 µM Fe(II)•BLM, 1 µM hairpin DNA-C<sub>f15</sub> and 1 µM denatured calf thymus DNA or 0.25 µM, 0.5 µM, 0.75 µM, 1 µM and 2 µM DNA 7 in 10 mM cacodylate buffer solution, pH 7.0, with 100 mM NaCl. The emission spectra were obtained following excitation at 310 nm at 25 °C.

In a separate experiment, the co-incubation of 1 µM random DNA with the 16-nucleotide hairpin DNA reduced the fluorescent peak to ~50% of the control peak, suggesting that
DNA 7, in comparison with the random DNA, has a stronger affinity to Fe(II)•bleomycin A₅, which makes bleomycin less available for the binding and subsequent cleavage of the 16-nucleotide hairpin DNA. The cleavage experiments involving radiolabeled DNA 7 and the 16-nucleotide DNA also displayed a higher order of DNA 7 cleavage with the increasing concentration of random DNA (Figure 3.4). The result has been summarized in Figure 3.5.

**Figure 3.4.** Competition Effects of Unlabeled Denatured Calf Thymus DNA on Fe(II)•BLM A₅ Mediated Cleavage of [5'-³²P]-End Labeled Hairpin DNAs 7 and 16-nt Hairpin DNA. (A) Lane 1, 640 μM (nucleotide concentration) radiolabeled DNA 7 alone; lane 2, 20 μM Fe²⁺; lane 3, 20 μM BLM A₅; lane 4, 20 μM Fe(II)•BLM A₅; lanes 5-10, 20 μM Fe(II)•BLM A₅ + 160, 320, 640, 1280, 2560, and 5120 μM (nucleotide
concentrations) unlabeled calf thymus DNA, respectively. (B) Lane 1, 160 μM (nucleotide concentration) radiolabeled 16-nt hairpin DNA alone; lane 2, 20 μM Fe$^{2+}$; lane 3, 20 μM BLM A$_5$; lane 4, 20 μM Fe(II)·BLM A$_5$; lanes 5-10, 20 μM Fe(II)·BLM A$_5$ + 40, 80, 160, 320, 640, and 1280 μM (nucleotide concentrations) unlabeled calf thymus DNA, respectively.

**Figure 3.5.** Comparison of the Effects of Unlabeled Calf Thymus DNA on the Cleavage of [5'-$^{32}$P]-End Labeled Hairpin DNA 7 and 16-Nucleotide DNA by Fe(II)·BLM A$_5$. The cleavage produced by Fe(II)·BLM A$_5$ on DNA 7 and 16-nucleotide DNA in the absence of the 16-nt hairpin DNA was defined as 100%.

In recent literature, two of the DNAs (DNA 7 and DNA 10) were also shown to be good substrates for bleomycin induced dsDNA cleavage.$^{56}$ In this study, DNA 7-4Me and DNA 11-5Me were designed by replacing 4 and 5 of the cytidines by 5'-methylcytidine; whereas DNA 10-Me had only one modification at C$_{13}$, which has been reported as a primary site for dsDNA recognition and cleavage (Figure 3.1).$^{56}$
Table 3.1. Inhibition of Fluorescence Emission by Hairpin DNAs\(^a\)

<table>
<thead>
<tr>
<th>hairpin DNA</th>
<th>binding specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 7</td>
<td>97±1</td>
</tr>
<tr>
<td>DNA 7-4Me</td>
<td>91±2</td>
</tr>
<tr>
<td>DNA 10</td>
<td>85±1</td>
</tr>
<tr>
<td>DNA 10-Me</td>
<td>20±3</td>
</tr>
<tr>
<td>DNA 11</td>
<td>97±1</td>
</tr>
<tr>
<td>DNA 11-5Me</td>
<td>13±3</td>
</tr>
</tbody>
</table>

\(^a\) The binding specificity (%) was calculated as the decrease in fluorescence intensity at maximum emission wavelength (455 nm) from no competitor (0%) through the reaction mixture without Fe\(^{2+}\) (100%). Standard errors were calculated based on three experiments under similar conditions.\(^79\)

As demonstrated in Figure 3.6, DNA 7-4Me and DNA 11-5Me containing methylated cytidines (Figure 3.1), on some characterized\(^54\) bleomycin cleavage sites, exhibited diminished binding affinity for bleomycin A\(_5\) comparing to their non-methylated counterpart, DNA 7. Whereas the relative binding affinity of DNA 7 was reduced moderately from 97% to 91% upon methylation of some of its reported cleavage sites, DNA 11-5Me, having all of the potential cleavage sites replaced by 5'-methylcytidine, exhibited more significant reduction of binding affinity, from 97% to 13% (Table 3.1). DNAs 7 and 11 have been shown to be strong binders of bleomycin A\(_5\) in previous studies and both of them are known to have multiple sites of bleomycin cleavage including putative dsDNA cleavage sites.\(^56\)
Figure 3.6. Fluorescence Emission Spectra of the 2’-Deoxyriboside of 4aminobenzo[g]quinazoline-2-one, Resulting from the Treatment of 16-nt Hairpin DNA C_{115} With Fe(II)•BLM in the Presence or Absence of 64-nt Hairpin DNAs. (A) Emission spectra derived from the treatment of 1 µM Fe(II)•BLM on 1 µM 16-nt hairpin DNA with or without 1 µM DNA 7 or DNA 7-4Me. The emission of 1 µM 16-nt hairpin DNA with BLM A_{5} was used as control. (B) Emission spectra derived from the mixture of 1 µM Fe(II)•BLM and 1 µM 16-nt hairpin DNA with or without 1 µM DNA 11 or DNA 11-5Me. The emission of 1 µM 16-nt hairpin DNA with BLM A_{5} was used as control.\(^{79}\)

The differential degree of binding affinity (Table 3.1) of DNA 7-4Me and DNA 11-5Me is thought to be dependent on the location of the sites of cytidine methylation; this is further studied by the similar experiments on DNA 10, as illustrated in Figure 3.7. DNA 10 being a comparatively weak binder of bleomycin\(^{57}\) and DNA 10-Me, containing
only one methylated cytidine on its previously characterized dsDNA cleavage site shows a dramatic change of binding affinity, from 85% to 20% (Figure 3.7).

**Figure 3.7.** Emission Spectra Following Admixture of 1 μM Fe(II)•BLM and 1 μM 16-nt Hairpin DNA With or Without 1 μM DNA 10 or DNA 10-Me. The emission of 1 μM 16-nt hairpin DNA with BLM A₅ was used as a control and considered as 100%. The differences between the control, without Fe(II), and the emissions of the 16-nucleotide DNA in a mixture with equimolar 64-nucleotide DNAs were the measures of the relative binding affinity of the hairpin DNAs.⁷⁹

To further study the effect of cytidine methylation on bleomycin binding, another hairpin DNA construct, DNA 7-5Me was designed by substituting C₁₇ of DNA 7-4Me by a 5-methylcytidine and binding affinity of the DNAs were compared (Figure 3.8).
**Figure 3.8.** Fluorescence Emission Spectra of the Profluorescent 16-nt Hairpin DNA C_{15} With Fe(II)•BLM in the Presence or Absence of One Equivalent of a 64-nt Hairpin DNA. Emission spectra obtained following admixture of 1 µM 16-nt hairpin DNA with 1 µM Fe(II)•BLM A_{5} with or without 1 µM DNA 7 or DNA 7-5Me. The emission of 1 µM 16-nt hairpin DNA following treatment with metal-free BLM A_{5} was used as a control. The emission spectra were obtained following excitation at 310 nm.\(^79\)
Unsurprisingly, DNA 7-5Me showed reduced affinity for bleomycin binding (Table 3.2) compared to DNA 7 and DNA 7-4Me, which supports the hypothesis that methylation on cytidine gradually decreases bleomycin binding to DNA.

Table 3.2. Inhibition of Fluorescent Emission by Hairpin DNAs

<table>
<thead>
<tr>
<th>hairpin DNA</th>
<th>binding specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 7</td>
<td>97±1</td>
</tr>
<tr>
<td>DNA 7-5Me</td>
<td>64±4</td>
</tr>
<tr>
<td>DNA 9</td>
<td>90±2</td>
</tr>
<tr>
<td>DNA 9-dU</td>
<td>95±1</td>
</tr>
<tr>
<td>DNA 10-Me</td>
<td>20±3</td>
</tr>
<tr>
<td>DNA 10-2Me</td>
<td>14±2</td>
</tr>
<tr>
<td>DNA 10-3Me</td>
<td>11±1</td>
</tr>
</tbody>
</table>

*The binding specificity (%) was calculated as the decrease in fluorescence intensity at maximum emission wavelength (455 nm) compared to the reaction having no competitor (0%) and the reaction mixture without Fe²⁺ (100%). Standard errors were calculated based on three experiments under similar conditions.*

3.2.2. DNA Methylation Results in Reduced Cleavage by Fe•BLM.

More interestingly, when bleomycin induced DNA cleavages of 5'-radiolabeled DNA 7 and DNA 10 was compared with their methylated counterparts an overall reduction of the intensity of cleavage bands was observed (Figure 3.9). The 5'-end labeled DNA 7, when subjected to bleomycin cleavage, resulted in seven notable cleavage bands, representing putative cleavage products (Figure 3.9A), similar to previously reported data. When incubated with 5 µM Fe(II)•bleomycin, the methylated
counterpart shows an overall reduction of the intensity of the cleavage bands, suggesting lower susceptibility of methylated DNAs for bleomycin induced cleavage (Figure 3.9B).
When DNAs were incubated with excess (10 µM) bleomycin, the cleavage bands involving sites of methylation (C₁₅ or C₁₃) or a site adjacent (A₁₂) to methylated cytidines displayed lower intensity compared to the sites far from methylated cytidines, such as C₁₇, T₁₀ and A₉. The percentage cleavage intensity at each site was measured by calculating the ratio of the intensity of the cleavage bands to the overall cleavage in each lane, represented by the summation of all cleavage bands in the individual lanes of Figure 3.9. The summary of cleavage intensities is illustrated in Figure 3.10. Although DNA 10 showed a reduction in cleavage intensity at the site of methylation (C₁₃) and the neighboring sites such as A₁₀ in a similar fashion (Figure 3.9C and D), more interestingly the cleavage at A₁₉, a site far from C₁₃, undergoes reduction of cleavage even when incubated with 5 µM bleomycin, suggesting a broader effect of methylation on bleomycin induced DNA cleavage, at least for some DNAs. A similar effect has been recorded when DNA 11 and DNA 11-5Me were subjected to 5 µM and 10 µM bleomycin A₅ treatment for 30 minutes. DNA 11 is also a tight binder as illustrated in Figure 3.6 with several possible double-strand DNA cleavage sites located within the variable region of the hairpin DNA, two of which are sites of coupled double-strand DNA cleavages (Figure 3.11). The overall cleavage of DNA 11-5Me, under a single hit kinetic condition, was diminished from 30% (DNA 11) to 17% at 5 µM Fe(II)•BLM treatment. The result is less dramatic at 10 µM bleomycin treatment (Figure 3.12).
Figure 3.10. Summary of the Cleavage Intensity at the Nucleotides in the Variable Region of DNA 7 and DNA 7-Me. (A) Comparison of 5 µM bleomycin A5 induced cleavage of nucleotides C17-A9 of DNA 7 and DNA 7-Me. (B) Comparison of 10 µM bleomycin A5 induced cleavage of nucleotides C17-A9 of DNA 7 and DNA 7-Me. The standard errors were calculated based on three experiments under similar conditions.
Figure 3.11. Analysis of Fe•Bleomycin Induced Double-Strand Cleavage Sites on Hairpin DNA 11. (A) Double strand cleavage of [5′-32P]-end labeled (lanes 1 and 2) and [3′-32P]-end labeled (lanes 3 and 4) 64-nucleotide hairpin DNA 11 by bleomycin A5. Lane 1, [5′-32P]-end labeled DNA alone; lane 2, 5 µM Fe(II)•BLM A5; lane 3, 5 µM Fe(II)•BLM A5; lane 4, [3′-32P]-end labeled DNA alone. (B) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [5′-32P]-end labeled DNA 11. Each lane (except lanes 1) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam-Gilbert G+A sequencing lane of [5′-32P]-end labeled DNA 11; lane 2, band 2a; lane 3, band 2b; lane 4, band 2c; lane 5, band 2d; lane 6, band 2e. (C) Sequencing gel analysis of bleomycin induced double-strand cleavage sites on [3′-32P]-end labeled DNA 11. Each lane (except lanes 1) corresponds to a numbered cleavage band, shown in (A). Lane 1, Maxam-Gilbert G+A sequencing lane of [3′-32P]-end labeled DNA 11; lane 2, band 3a; lane 3, band 3b; lane 4, band 3c; lane 5, band 3d; lane 6, band 3e. (D) Summary of Fe•bleomycin induced double-strand cleavage sites on hairpin DNAs 11. Orange bases indicate randomized region of the original hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to couple double-strand cleavage events whereas red arrows indicate non-coupled double-strand cleavage events, which results from two independent single-strand cleavages on opposite strands. Nucleotides colored in red indicate primary sites of double-strand cleavage.79
**Figure 3.12.** Bleomycin Cleavage of 5’-Labeled DNA 11 and DNA 11-5Me. (A) Sequence Selective Cleavage of DNA 11 by BLM A₅. Lane 1, DNA 11 alone; lane 2, 5 μM Fe(II)•BLM A₅; lane 3, 10 μM Fe(II)•BLM A₅; lane 4, G+A lane. (B) Sequence selective cleavage of DNA 11-5Me by BLM A₅. lane 1, DNA 11-5Me alone; lane 2, 5 μM Fe(II)•BLM A₅; lane 3, 10 μM Fe(II)•BLM A₅; lane 4, G+A lane.

### 3.2.3. Characterization of Double-Strand Cleavage of Methylated DNAs.

Beside the change in bleomycin-induced cleavage, it was surprising that DNA 10-Me having a 5-methylcytidine at C₁₃ showed a shift of bleomycin induced dsDNA cleavage. As reported in a previous study, DNA 10 shows three pairs of dsDNA cleavages (Figure 3.13). Methylation of one of the primary sites of dsDNA cleavage resulted in reduced cleavage at C₁₃-G₅₂ with an obvious reduction of overall cleavage of
the DNA (Figure 3.14A). The histogram shown in Figure 3.14B illustrates that the percentage dsDNA cleavage at C\textsubscript{13}-G\textsubscript{52} goes down significantly (from \(~10\%\) to \(~1.5\%).

![Diagram of DNA 7, 10, and 11]

**Figure 3.13.** Summary of Fe•Bleomycin-Induced Double-Strand Cleavage Sites on DNAs 7, 10, and 11. Orange bases indicate randomized region of original hairpin DNA library. Arrows of the same shape and color indicate paired cleavages. Black arrows correspond to coupled double-strand cleavage events whereas red arrows indicate non-coupled double-strand cleavage events, resulting from two independent single-strand cleavages on opposite strands. Nucleotides colored in red indicate primary sites of coupled double-strand DNA cleavage.\textsuperscript{79}
Figure 3.14 Patterns of the Double-Strand Cleavage of DNA 10 and DNA 10-Me. (A) Native polyacrylamide gel representing double-strand cleavage of [5′-32P]-end labeled (lanes 2 and 4) and [3′-32P]-end labeled (lanes 1 and 3) 64-nucleotide hairpin DNA 10 (lanes 1-2) and DNA 10M (lanes 3-4) by Fe(II)•BLM A5. (B) Histogram representing shift of dsDNA cleavage intensity from C13-G52 site to the neighboring sites.79

Two other constructs, DNA 10-2Me and DNA 10-3Me, having two and three cytidine substituted by 5′-methylcytidine (Figure 3.15), when compared for bleomycin binding affinity showed a reduction of inhibition from 20% (DNA 10-Me) to 14% and...
11%, respectively (Figure 3.16). The fluorescence inhibition data, which reflect the binding affinity of hairpin DNAs, are summarized in Table 3.2. Additionally, when DNA 10-2Me and DNA 10-3Me were subjected to 5 μM bleomycin A₅ treatment under single hit kinetic conditions, overall dsDNA cleavage was reduced to 5% and 3%, respectively (Figure 3.17). Compared to DNA 10-Me (Figure 3.14), hairpin DNA 10-2Me demonstrated an overall reduction of dsDNA cleavage of 50% with the addition of a methylated cytidine at C₅₃, another putative bleomycin cleavage site. Consistent with the trend, DNA 10-3Me showed even less dsDNA cleavage.

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**Figure 3.15.** DNA 10 Constructs With Substitutions at C₁₃ Alone Forming DNA 10-Me; C₁₃ and C₅₃ Forming DNA 10-2Me; C₁₃, C₁₁ and C₅₃ Forming DNA 10-3Me, Respectively.
Figure 3.16. Fluorescence Emission Spectra of the Profluorescent 16-nt Hairpin DNA C_{15} With Fe(II)•BLM in the Presence or Absence of One Equivalent of a 64-nt Hairpin DNA. Emission spectra obtained following treatment of 1 µM 16-nt hairpin DNA with 1 µM Fe(II)•BLM A_{5} with or without 1 µM DNA 10 and methylated variants of DNA 10, DNA 10-2Me and DNA 10-3Me. The emission of 1 µM 16-nt hairpin DNA following treatment with metal-free BLM A_{5} was used as a control. The emission spectra were obtained following excitation at 310 nm.79

Table 3.3. Percentage of DNA Cleavage of Hairpin DNAsa

<table>
<thead>
<tr>
<th>hairpin DNA</th>
<th>percentage cleavage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 7</td>
<td>35±3</td>
</tr>
<tr>
<td>DNA 7-4Me</td>
<td>16±2</td>
</tr>
<tr>
<td>DNA</td>
<td>26±4</td>
</tr>
<tr>
<td>DNA 10-Me</td>
<td>16±3</td>
</tr>
<tr>
<td>DNA 11</td>
<td>33±3</td>
</tr>
<tr>
<td>DNA 11-5Me</td>
<td>15±4</td>
</tr>
<tr>
<td>DNA 9</td>
<td>20±1</td>
</tr>
<tr>
<td>DNA 9-dU</td>
<td>27±2</td>
</tr>
</tbody>
</table>

aThe percentage cleavage for 5 µM bleomycin treatment was calculated as the ratio of the intensity of cleavage bands to the overall intensity of the hairpin DNA loaded in each well of denaturing polyacrylamide gel. Standard errors were calculated based on three experiments under similar conditions.79
Figure 3.17. Patterns of the Double-Strand Cleavage of DNA \textbf{10-2Me} and DNA \textbf{10-3Me}. (A) Native polyacrylamide gel showing bands resulting from double-strand cleavage of 5'-32P-end labeled (lanes 1 and 3) and [3'-32P]-end labeled (lanes 2 and 4) 64-nucleotide hairpin DNA \textbf{10-2Me} (lanes 1 and 2) and DNA \textbf{10-3Me} (lanes 3 and 4) by 5 \(\mu\)M Fe(II)•BLM A5 for 1 minute. (B) Histogram illustrating the overall decrease of double-strand DNA cleavage intensity of C_{13-G52} and C_{13-G52} sites upon methylation of C_{53} (DNA \textbf{10-2Me}) and C_{11} (DNA \textbf{10-3Me}) in addition to C_{13}.79

3.2.4. Structural Basis for Diminished Binding and Cleavage of DNAs Containing Methylated Cytidines.

To investigate the effect of DNA methylation on BLM interaction, hairpin DNA 9 was chosen. The DNA has two putative (5'-GT- 3') bleomycin cleavage sites. A construct was made with substituting T_{13} and T_{16} by deoxyuridine and the effect of bleomycin binding was studied by employing fluorescent inhibition assay (Figure 3.18). The substitution of deoxyuridine, lacking a methyl group in comparison with thymidine,
Figure 3.18. Fluorescence Emission Spectra of the Profluorescent 16-nt Hairpin DNA C_{115} With Fe(II)•BLM in the Presence or Absence of One Equivalent of a 64-nt Hairpin DNA. Emission spectra obtained following treatment of 1 µM 16-nt hairpin DNA with 1 µM Fe(II)•BLM A_{5} with or without 1 µM DNA 9 or DNA 9-dU. The emission of 1 µM 16-nt hairpin DNA following treatment with metal-free BLM A_{5} was used as a control. The emission spectra were obtained following excitation at 310 nm. Increased the binding affinity of bleomycin from 90% (DNA 9) to 95% (Table 3.2). This increased binding of bleomycin to DNA 9-dU was also reflected in Fe(II)•bleomycin induced cleavage of the hairpin DNA. Under the single hit kinetic condition, the overall cleavage of DNA 9-dU was increased to 27% from 20% in DNA 9. The increased cleavage and binding of DNA 9-dU was believed to be linked with the substitution of ‘T’ s in DNA 9 by deoxyuridine at the bleomycin cleavage sites (Figure 3.19 and Table 3.3). The bleomycin cleavage of DNA 9 and DNA 9-dU was further investigated in a
time dependent manner at reduced temperature (0 °C), which showed the increase of cleavage of DNA 9-dU compared to DNA 9 (Figure 3.20).

**Figure 3.19.** Sequence Selective Cleavage of DNA 9 and DNA 9-dU by BLM A₅. (A) Lane 1, DNA 9 alone; lane 2, 1 μM Fe(II)•BLM A₅; lane 3, 5 μM Fe(II)•BLM A₅; lane 4, 10 μM Fe(II)•BLM A₅; lane 5, G+A lane. (B) Lane 1, DNA 9-dU alone; lane 2, 1 μM Fe(II)•BLM A₅; lane 3, 5 μM Fe(II)•BLM A₅; lane 4, 10 μM Fe(II)•BLM A₅; lane 5, G+A lane.
Figure 3.20. Time Dependent, Sequence Selective Cleavage of DNA 9 (A) and DNA 9-dU (B) by 5 µM BLM A₅ at 0 °C. (A) Lane 1, 5 min; lane 2, 10 min; lane 3, 15 min; lane 4, 20 min; lane 5, 25 min; lane 6, 30 min; lane 7, G+A lane. (B) Lane 1, 5 min; lane 2, 10 min; lane 3, 15 min; lane 4, 20 min; lane 5, 25 min; lane 6, 30 min; lane 7, G+A lane. (C) Histogram representing time dependent enhanced cleavage of DNA 9-dU.⁷⁹
3.3. Discussion

Despite early evidence from the Hecht laboratory supporting the effects of DNA methylation on DNA cleavage by bleomycin,\(^{80,81}\) no systematic analysis on the effects of DNA methylation on DNA binding or cleavage has been reported. In this study we focused on factors that make DNAs amenable to high affinity binding by bleomycin under conditions of limited drug usage, suggesting the need to consider the role of naturally occurring DNA modifications. Cytidine methylation is known to affect nucleosome structure\(^{82}\) and alter gene expression, and the methylation pattern of DNA changes in many cancers, making it obvious to consider DNA methylation as one important factor influencing the action of bleomycin in a therapeutic setting.

In recent publications, we have provided evidence suggesting that at low concentrations, bleomycin has a strong affinity for selected DNAs, and that these are likely to be bound selectively to bleomycin in a mixture of DNAs.\(^{53,57,58,83,84}\) Further, these tight binding DNAs undergo a newly characterized type of double-strand cleavage, which may result in double-strand gaps in duplex DNA.\(^{51,54}\) Given the selective targeting of tumor cells by BLM,\(^{34,85,86}\) the double-strand gaps in the genome of tumor cells may provide the basis for the cytotoxic effect of bleomycin. While DNA methylation within the human genome is abundant in both normal and cancer cells, the alteration of DNA methylation in cancer cells may plausibly provide additional insights into characteristics of the targets responsible for selective tumor cell killing by bleomycin.

Not all cytidine methylations had the same impact on bleomycin binding to DNA. Methylation at a single position (C\(_{13}\)) in DNA 10 decreases binding of Fe(II)•BLM A\(_5\) considerably but the effect was not greatly enhanced by additional methylations at C\(_{53}\)
and C_{11} (Table 3.2). This bis-methylated species (DNA 10-2Me) demonstrated the same BLM binding specificity as DNA 11-5Me with five methylated cytidines (Table 3.2). In contrast, DNA 7-4Me having four methylated cytidines had little effect on the binding of that hairpin DNA, although DNA 7 and DNA 11 exhibited similar affinities for Fe(II)•BLM A_5 (Table 3.1). An addition of a fifth methyl group to DNA 7-4Me at a known site of double-strand DNA cleavage (C_{17}, Figure 3.8 and Table 3.2) afforded DNA 7-5Me, showed enhanced specificity of bleomycin binding. This suggested that the site of methylation is important for its effect on Fe•BLM binding.

The same conclusion was reached by observing the effects of cytidine methylation on Fe•BLM mediated DNA cleavage of DNA 10. As shown in Figures 3.7 and 3.9, methylation at C_{13} led to an overall decrease of DNA cleavage (Table 3.3) with an additional shift of the observed cleavage focus to sites distant from the site of methylation. This implied that methylation could possibly shift the preferred site of DNA binding.

There are many examples of the site selective cleavage of BLM involving both DNA and RNA. When excess Fe(II)•BLM is used with the combination of the B-form DNAs, the major cleavage sites involve 5′-GT and 5′-GC sequences,^{17,43} and the 5′-GPy sequences also appear as the primary sites of cleavage in a coupled double-strand DNA cleavage event.^{54} It is believed that Fe•BLM cleavage of DNA is preceded by binding, which limits cleavage to a subset of DNA and RNA sites bound effectively by Fe•BLM.^{85} The relatively wide and shallow minor groove of B-form DNA at 5′-GT and 5′-GC sequences may plausibly favor binding by Fe•BLM. This was further supported by the fact that Fe•BLM cleaves the duplex domain of a DNA triplex at the junction of 5′-
duplex-triplex, where the minor groove is wider and shallower than in canonical B-form DNA, as suggested by computational modeling. Similarly, the cleavage of transfer RNAs by Fe•BLM was disproportionately enhanced at the junction of single and double-strand regions, where a structure similar to the minor groove of DNA is expected to be relatively wide, and shown to be sensitive to small changes in tRNA structure.

As summarized in Tables 3.1 and 3.2, cytidine methylation in hairpin DNAs led to decreased Fe•BLM binding affinity and diminished DNA cleavage (Table 3.3). Bleomycin mediated DNA cleavage is initiated with the abstraction of a hydrogen atom from the C4’ position of a deoxyribose sugar in the backbone of the minor groove of DNA. Given that the methyl group of 5-methylcytidine projects outwards from the major groove, the decrease of DNA binding and cleavage may not be a direct result of the steric interaction between the methyl group and Fe•BLM. However, it seems logical to believe that introduction of one or more methyl groups on the DNA major groove might compress the DNA minor groove to relieve the induced steric interactions, which ultimately affects Fe•BLM binding and cleavage in the minor groove. The reported decrease in flexibility in methylated DNA may also play a key role in DNA binding and cleavage by Fe•BLM.

Assuming that the introduction of methyl groups in the major groove of DNA is the source of reduced cleavage in the DNA minor groove, it is logical to expect that the removal of a methyl groups might increase Fe•BLM binding and cleavage. DNA binding and cleavage studies involving hairpin DNAs have been carried out in DNAs having (per)methylated major grooves, since all of the DNAs in the original hairpin DNA library had thymidine residues within the randomized 8 base pair region. Hairpin DNA had
five thymidine residues and two of which (T₁₃ and T₁₆) are primary sites of double-strand cleavage. Replacement of these thymidines with deoxyuridines afforded DNA 9dU, which augmented the binding affinity for Fe•BLM (Table 3.2) and demonstrated increased DNA cleavage products (Table 3.3). Even though the differences in cleavage involving DNAs 9 and 9-2dU were not large at room temperature, a 2-3 fold increase in cleavage was noted for DNA 9-2dU after 25-30 minutes at 0 °C, where the rate of DNA cleavage by Fe•BLM is slower.

The modulation of DNA minor groove width has been reported several times in the past, as a consequence of the binding of proteins and hairpin polyamide DNA binders to the DNA major groove. The present results offers an idea that Fe•BLM is sensitive to the dimensions of the minor groove to distinguish even small changes in geometry associated with the introduction of a methyl group to pyrimidines in the DNA major groove.

In conclusion, we have demonstrated that methylation of cytidine not only reduces the overall cleavages mediated by bleomycin but also changes the dsDNA cleavage pattern. Cytidines, being a part of highly conserved CpG sequences, have direct involvement in epigenetic gene regulation, developmental processes and cancer biogenesis.
Figure 3.21. Overall Change of the Cytidine Methylation Pattern in Cancer. Cancer is marked by global hypomethylation of DNA, making it more susceptible to bleomycin induced DNA degradation.

The non-methylated and methylated hairpin DNAs used in our study hypothetically mimicked DNA methylation conditions observed in cancer cells. We demonstrated that compared to their methylated counterparts hypo-methylated DNA is highly susceptible to bleomycin-induced overall site-specific degradation (Figure 3.9) and dsDNA cleavages (Figure 3.14). This suggests an increased lethal effect of bleomycin in cancer cells. Bleomycin, which has been reported to be a minor groove binder,\(^9\) also shows a variable degree of reduction of binding to the methylated DNAs as a result of the possible local structural change due to methylation.\(^10\) The compromised binding and subsequent reduction of cleavage induced by bleomycin may be derived from the local pinching of the minor groove, without disrupting the overall structure of DNA at the site of methylation.\(^101,102\)
Summarizing our findings in Figure 3.20, we posit that the potency of bleomycin as an antitumor agent is affected by DNA methylation, providing a model to explain its possible enhanced binding and degradation of DNA in cancer cells.

3.4. Experimental

3.4.1. Materials.

T4 polynucleotide kinase was purchased from New England Biolabs. Recombinant terminal deoxynucleotidyl transferase was obtained from Roche Applied Science. Radiolabeled nucleotides [γ-32P]-ATP and [α-32P]-cordycepin were purchased from Perkin Elmer Life Sciences. Fe(NH₄)₂(SO₄)₂•6H₂O and Chelex 100 were from Sigma Aldrich. All the synthetic oligonucleotides including the hairpin DNAs were obtained from Integrated DNA Technologies, Inc. The 16-nucleotide hairpin DNA-Cf15 was purchased from Chemgenes using nucleotide analogue Cf15 synthesized by M. P. Alam. The 64 nucleotide hairpin DNA 11 was obtained from Chenhong Tang through a rigorous selection process with the goal of obtaining hairpin DNAs tightly bound to Fe(III)•BLM A₅.

3.4.2. End Labeling and Purification of Hairpin DNAs and Their Methylated Counterparts.

The hairpin DNAs were 32P-end labeled using a combination of [γ-32P]-ATP and T4 polynucleotide kinase or [α-32P]-cordycepin and terminal deoxytransferase at the 5’ and 3’-ends, respectively. Ten pmol of 64-nucleotide hairpin DNAs were [5’-32P]-end labeled by incubation with 20 units of T4 polynucleotide kinase and 0.06 mCi [γ-32P]-
ATP (specific activity 6000 Ci (222 TBq)/mmol) in 50 μL (total volume) of 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl₂ and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by DNA purification by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h. The [3ʹ-32P]-end labeling was done by incubating 10 pmol of hairpin DNA with 20 units of terminal deoxynucleotidyl transferase and 0.06 mCi [α-32P]-ATP (specific activity 6000 Ci (222 TBq)/mmol) in 50 μL (total volume) of 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl₂, 10 mM CoCl₂ and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by purification of DNA by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h.

3.4.3. Double-Strand DNA Cleavage of Hairpin DNA 10, 10-Me, 10-2Me and 10-3Me.

Bleomycin cleavage of 5ʹ and 3ʹ-end labeled hairpin DNAs was performed by incubating the hairpin DNA (~30000 cpm) with 5 μM Fe²⁺ and 5 μM bleomycin A₅ at 25 °C for 30 min in a 10 μL 10 mM Na cacodylate, pH 7.0 solution containing 2 mM MgCl₂. Two μL of native gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 40% D-sucrose were added to the reaction mixture, which was separated on a 20% native polyacrylamide gel (200 V, 16 h, 4 °C). Double-strand cleavage sites were identified by visualizing co-migrating bands, using a phosphorimager.
3.4.4. Maxam-Gilbert Sequencing Reaction.\textsuperscript{65}

Ten µL of 5’ and 3’-end labeled DNAs (~50000 cpm) were incubated with 25 µL of formic acid at 25 °C for 5 min. The reactions were mixed with 200 µL of 0.3 M sodium acetate, 0.1 mM EDTA, and 25 µg/mL \textit{E. coli} tRNA. The resulting solutions were immediately mixed with 700 µL ethanol to precipitate the DNAs. The DNA pellets were washed twice with 70% ethanol, and the pellets were resuspended in 75 µL of 10% piperidine. The reaction mixtures were incubated at 90 °C for 30 min and then chilled on ice. The cooled supernatants were concentrated under diminished pressure. The DNA pellets were washed with small amounts of water to remove residual piperidine and mixed with denaturing loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue and 1% xylene cyanol. The combined solutions were heated at 90 °C for 10 min and used as the sequencing lanes to compare [5’-\textsuperscript{32}P]-end and [3’-\textsuperscript{32}P]-end labeled DNAs on denaturing polyacrylamide gels.

3.4.5. Sequence-Selective Cleavage of Radiolabeled Hairpin DNAs by BLM A\textsubscript{5}.

[5’-\textsuperscript{32}P]-end-labeled hairpin DNA 7, DNA 7-Me, DNA 10 and DNA 10-Me (~50000 cpm) were incubated with 5 µM or 10 µM Fe•BLM A\textsubscript{5} in a 5 µL reaction, containing 10 mM Na cacodylate buffer, pH 7.0, for 30 min. Five µL of denaturing gel loading buffer containing 98% formamide, 2 mM EDTA, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol were added to the reaction mixture. The resulting solutions were heated at 90 °C for 10 min followed by chilling on ice. Five µL of each sample was loaded onto a denaturing gel (16% polyacrylamide and 7 M urea) along with 2 µL of 5’-\textsuperscript{32}P and [3’-\textsuperscript{32}P]-end labeled Maxam-Gilbert sequencing lanes to determine the
sequences of the cleavage sites. The gels were run at 50 W for 2.5 h. The gels were visualized using a phosphorimager.

### 3.4.6. Fluorescence Competition Analysis of Hairpin DNAs.

Five µM 64-nucleotide hairpin DNA solutions were incubated with 5 µM bleomycin A₅ in 10 mM sodium cacodylate buffer solution, pH 7.0, at room temperature for 20 min. The resulting solution was mixed with equimolar 16-nucleotide hairpin DNA having Cᵢ at cytidine15 (hairpin DNA-Cᵢ₁₅) and incubated at room temperature for 1 min. Freshly prepared Fe(NH₄)₂(SO₄)₄ was added to the solution to a final concentration of 1 µM. In the final solution 16-nucleotide hairpin DNA, 64-nucleotide hairpin DNAs, bleomycin and Fe²⁺ were all present at 1 µM concentration. The solution was maintained at room temperature for 30 min. The same volume of buffer solution was added to the control sample without Fe²⁺ and 64-nucleotide hairpin DNA. The fluorescence emission was measured at 25 °C. The samples were excited at 310 nm, and the emission signal was collected from 400 to 550 nm using an excitation slit width of 10 nm and an emission slit width of 10 nm.
CHAPTER 4
EVALUATION OF THE INTERACTION BETWEEN BCL2 PROMOTER ELEMENT
AND INDIVIDUAL DOMAINS OF hnRNP LL, LEADING TO A STRUCTURAL
CHANGE IN DNA

4.1. Introduction

DNA secondary structures are known to play important roles in complex cellular processes. Studies have shown that the presence of secondary structures is fairly common in functionally important regions in the genome. One such widely studied secondary structure is the G-quadruplex, which is found in several promoter sites, 5′ UTRs and telomeric regions. Similar to G-quadruplexes, another interesting DNA structural element is the cytidine rich i-motif. Unlike the G-quadruplex, the i-motif structure is highly flexible and several factors such as pH and heat can play vital roles in folding of DNA, leading to specific i-motif structures. Analogous to their structural counterpart G-quadruplexes, i-motifs are also abundant in the promoter regions of certain genes. One of such important gene is BCL2 (B-cell lymphoma gene-2).

The product of the BCL2 gene is an anti-apoptotic protein, classified as an oncogene. Overexpression of the BCL2 gene prolongs cell survival, linking it to the development of cancer. Previous studies have demonstrated that the C-rich upstream promoter region of BCL2 forms a highly dynamic i-motif structure, which can act as an transcriptional switch for the expression of the BCL2 gene. Recent findings further reveal that the BCL2 i-motif can be a potential target for small molecules and, more interestingly, for transcription factors such as hnRNP LL.
Figure 4.1. Sequence of hnRNP LL With Four Interactive Domains.

The hnRNP LL, homologous to hnRNP L with more than 50% sequence similarity in certain species, belongs to a class of small RNA binding proteins. Unlike hnRNP L and other proteins of that family, the structure of hnRNP LL has not been fully characterized. It is known that the protein has four functional domains, RRM1-4. Among these domains, RRM1 and RRM2 are separated by a short sequence of amino acids, whereas domains 3 and 4 are connected by a linker region (Figure 4.1). According to the hypothesis set forth in previous studies, two of the hnRNP domains (RRM1 and RRM2) are more likely to recognize and bind the BCL2 i-motif, and RRM3 and RRM4 could possibly play an important role in unwinding and stabilizing the downstream i-motif structural changes and engaging other factors to initiate transcription (Figure 4.2). In this study, we focused on the specific interactions of the domains of hnRNP LL and the BCL2 i-motif DNA, which may give us insights into the biological role of structurally complex DNAs. More specifically, understanding the interaction of BCL2 promoter and
transcription factors such as hnRNP LL could reveal the pattern of BCL2 gene regulation and help us to design peptides or peptide mimics to target complex DNA regions.

Figure 4.2. BCL2 Promoter i-Motif Sequence and Structures of Individual Domains of hnRNP LL. (A) Interchangeable i-motif and hairpin structure of BCL2 promoter element. (B) Proposed mechanism of BCL2 gene regulation by the interaction of Complex i-motif structure and transcription factor hnRNP LL. The binding interaction of hnRNP-LL domains with i-motif unfolds the complex structure and facilitates the downstream transcription.

4.2. Results

4.2.1. RRM Constructs.

To understand the individual interactions of the four domains of hnRNP LL and the BCL2 i-motif, the domains were cloned in pET 28a vectors and expressed separately using E. coli BL21-DE3 cells. Domains RRM1 or RRM2, and RRM3-4, were constructed with an additional 15 to 20 amino acids on both the C and N termini of the construct, flanking the sequence optimized for expression in E. coli (Figure 4.1). The genes were equipped with a Strep-tag at the C-terminus for streptactin mediated protein purification.
The domain structures as illustrated in Figure 4.3 were determined by sequence homology interpretation based on other well characterized homologous proteins of the family.

![Predicted Structures of RRM1, RRM2 and RRM3-4](image)

**Figure 4.3.** Predicted Structures of RRM1, RRM2 and RRM3-4 Based on Sequence H-Homology With Other Members of That Protein Family.

The three-dimensional structure of RRM1 was modeled with reference to the solution structure of N-terminus mouse protein BAB28521 (PDB ID: 1WEX). Similarly, the structure of RRM2 was predicted in reference with the solution structure of mouse hnRNP LL RRM2 (PDB ID: 2E5I), whereas RRM3-4 was modeled based on the crystal structure of human hnRNP L (PDB ID: 3TO8).

**4.2.2. Study of the Binding Interaction Between Domains and i-Motif.**

The binding interactions between each of the three constructs containing the four domains of hnRNP LL and the i-motif were studied by employing a gel shift assay. As postulated in previous studies, RRM1 and RRM2 showed strong binding to the DNA (Figure 4.4A and B) when the DNA to hnRNP LL domains molar ratio was varied from 1 to 8 equivalents. Furthermore, both domains were shown to interact with the i-motif.
DNA with varying affinities. The histograms in Figure 4.4C and D illustrate that at 8 molar equivalents concentration both the domains bind to DNA to the extent of >50%, when incubated at room temperature (Table 4.1). Interestingly, RRM3-4 (Figure 4.1) appeared to be a weak binder of the i-motif DNA (Figure 4.5 and Table 4.2). Varying concentrations of RRM3-4 (2.5 to 100 molar equivalents), when incubated with 1 equivalent of i-motif, showed less than 50% binding, even using 25 equivalents of the RRM domains. The gel reveals the presence of two protein–DNA complexes, labeled as complex 1 and complex 2 (Figure 4.5A), suggesting a more complex interaction between domain 3-4 of hnRNP LL and the i-motif.

Figure 4.4. Binding Interaction Study of RRM1, RRM2 and i-Motif DNA. (A & B) binding of RRM2 and RRM1 (1, 2, 4 and 8 molar eq.) with i-motif DNA, respectively. (C & D) Histogram showing percentage of DNA–protein complex formation with varying concentrations of RRM2 and RRM1, respectively. The Y-axes indicate percentage DNA binding with hnRNP domains.
Table 4.1. Percentage Binding of i-Motif by RRM1 and RRM2<sup>a</sup>

<table>
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<th>RRM1</th>
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<th>RRM2</th>
<th></th>
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<tbody>
<tr>
<td>eq. of protein to DNA</td>
<td>% binding</td>
<td>eq. of protein to DNA</td>
<td>% binding</td>
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<td>8</td>
<td>73</td>
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<sup>a</sup>The percentage binding of protein and DNA was calculated as the percentage intensity of the protein–DNA complex band relative to the total DNA in each lane.

In addition, both of the domains were shown to have a stronger affinity toward the i-motif DNA compared to calf thymus DNAs. The specificity of the binding interaction was studied with varying concentrations of calf thymus DNA (0.25 to 1 molar equivalent) in mixture with one equivalent of i-motif DNA and domains 1 or 2

Figure 4.5. Binding Affinity Study of RRM3-4 and i-motif DNA. (A) binding affinity of RRM3-4 (2.5, 5, 10, 20, 25, 50 and 100 molar eq.) for i-motif DNA. (B) Histogram showing percentage of DNA-protein complex formation with a varying concentration of RRM3-4.
(Figure 4.6A and B). In the presence of the equivalent concentration of a random competitor, both of the domains showed more than 50% binding interaction when incubated with equimolar substrate, i-motif DNA (Figure 4.6C, D and Table 4.3).

**Table 4.2.** Percentage Binding of i-Motif by RRM3-4

<table>
<thead>
<tr>
<th>eq. of RRM3-4 to i-motif</th>
<th>% to total i-motif</th>
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<tbody>
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</tr>
<tr>
<td>100</td>
<td>39</td>
</tr>
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</table>

*The percentage complex formation between protein domain and i-motif was calculated as the percentage intensity of the bands indicating complex formation relative to the total DNA in each lane.*

**4.2.3. Structural Change of i-Motif Following the Binding With RRM*s.*

Previous investigations suggested that the interaction between the domains and the i-motif is likely to unwind the complex secondary structure, which may result in transcription initiation from the promoter region of the *BCL2* gene. In this study, the structural change of *BCL2* i-motif has been studied while interacting with the domains of hnRNP LL, such as RRM1 and RRM2, by employing a bromine-footprinting technique (section 4.4.4). As observed in Figure 4.7, the i-motif DNA shows a differential rate of bromination on cytidine nucleotides in the SII and SIII regions of i-motif, followed by cleavage of DNA at brominated nucleotides. The variation of the extent of bromination is attributed to the complex structure of the i-motif, which restricts the accessibility of
cytidine for bromination. The i-motif shows unwinding of the complex structure at higher pH, with a transition pH at 6.6. It is evident from Figure 4.7 that when i-motif DNA was treated with RRM1 and RRM2 at pH 6.6, the accessibility of nucleotides for bromination changed dramatically, suggesting the conversion of the i-motif structure to a less complex DNA structure. A similar experiment done at pH 7.0 (Fig 4.8), which has a higher concentration of linear DNA, does not show any evidence of a further change in structure.
Table 4.3. Percentage Binding of i-Motif by Domains With Varying Random DNA\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>RRM1</th>
<th></th>
<th>RRM2</th>
<th></th>
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<tbody>
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<td>eq. of</td>
<td>eq.</td>
<td>%</td>
<td>eq.</td>
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\textsuperscript{a} The percentage binding of protein and DNA was calculated as the percentage intensity of the protein-DNA complex band to the total DNA in each lane.

Figure 4.7. Analysis of Structural Change in BCL2 i-Motif as a Result of Binding With RRM1 and RRM2. (A) structural change of i-motif observed in bromine footprinting
assay at pH 6.6. Lane 1, Maxam–Gilbert G+A lane; lane 2, DNA alone; lane 3, DNA + RRM1; lane 4, DNA + RRM2. (B) i-motif structure showing C-rich SII, SIII and loop L3, which shows significant changes upon bromine-footprinting assay.

The summary of the susceptibility of nucleotides, spanning C27 to C15, to Br2 induced cleavages, upon incubation with RRM1 and RRM2 at pH 6.6, reveals that stems 2 and 3 of the i-motif become more accessible to bromine, after the binding of the hnRNP LL domains, which results in at least partial relaxation of the complex i-motif structure (Figure 4.9).

**Figure 4.8.** Br Footprinting of i-Motif in the Presence or Absence of RRM1 and RRM2 at pH 7.5. Lane 1, Maxam–Gilbert G+A lane; lane 2, DNA alone; lane 3, DNA + RRM1; lane 4, DNA + RRM2; lane 5, DNA alone.
The i-motif structural change was studied by analyzing the CD spectra of the DNA in the presence of RRM1 and RRM2 (Figure 4.10). As noted previously, the BCL2 i-motif has a characteristic CD spectrum with a distinct peak at 286 nm. This peak decreases when the CD spectrum is monitored at higher pH, compared to a more acidic pH, suggesting a shift of equilibrium between two or more forms of the i-motif (Figure 4.2). As established in previous studies, the complex i-motif structure exists predominantly at lower pH, and decreases gradually with an increase in pH. As shown in Figure 4.10, the peak at 286 nm is decreased with the addition of RRM1 and RRM2, which suggests a significant structural change of i-motif when it interacts with the specific domains of the protein.

**Figure 4.9.** Histogram Resulting from Bromine Footprinting Assay Represents Structural Changes in the Region Between C27 to C15, Encompassing C-Rich SII, SIII and Loop L3 of i-Motif Due to the Binding Interaction With RRM1 and RRM2. The individual lines represent the trend of radioactive intensity of the cleavage bands at each nucleotides in SII, SIII and L3 regions of i-motif. The Y-axis indicates the absolute intensity of the cleavage bands as directly related to the availability of the individual nucleotides for bromination.

The change in the CD spectrum was less prominent when the spectral analysis of the hnRNP LL domains and the i-motif interaction was examined at higher pH, such as pH 7.5 (Figure 4.11). The lower reduction of the peak is attributed to the absence of the i-
motif structure, which is replaced by the unstructured DNA at higher pH, as suggested in earlier literature.\textsuperscript{113}

**Figure 4.10.** CD Spectra of i-Motif With Various Concentrations of RRM1 and RRM2, Suggesting Structural Change Upon Binding. (A) CD spectra of i-motif DNA at pH 6.6 with varying concentrations of RRM1. (B) CD spectra of i-motif DNA at pH 6.6 with varying concentrations of RRM2.

### 4.2.4. Sequence Based Binding Between i-motif and RRM\textsubscript{s}.

A computer simulation based on homologous protein hnRNP L, predicted the amino acids in RRM1 which may take part in substrate (i-motif) binding. A thorough binding interaction study and kinetic analysis was envisioned, employing the FRET
technique. In previous studies, the lateral loops 1 and 2 of the i-motif DNA (Figure 4.2) were found to interact with RRM1 and RRM2. In order to determine which region of the i-motif interacts with RRM1 and RRM2, several modified i-motif DNAs were synthesized (Figure 4.12) and their binding interactions with RRM1 and RRM2 were studied (Figure 4.13).

![Figure 4.11. CD Spectra of i-Motif and RRM1 (A), RRM2 (B) Interaction at pH 7.5.](image)

The CD spectra of the modified i-motifs reveals that a point mutation in the C-rich i-motif stem did not alter the equilibrium between the i-motif structures, which is evident in a small reduction of the i-motif peak at 286 (Figure 4.12A). Interestingly, the mutations in the loop structures of the DNA reveal that the nucleotide sequences of the L1 and L2 loops are essential for i-motif structure whereas loop L3 plays a less important role.
role in the stabilization of the i-motif (Figure 4.12B). As observed in Figure 4.13, the
mutations in loop structure decrease the binding of the i-motif to protein, whereas
structural changes, as a result of the point mutations, in the C-rich region of the i-motif
did not effectively change the binding interaction. This suggests, in full agreement with
the previous report,\textsuperscript{113} that the lateral loops flanking the C rich i-motif structures are
important for RRM1 and RRM2 binding.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_12.png}
\caption{Circular Dichroism Analysis of BCL2 i-Motif Mutants. (A & B) Change in CD spectra of BCL2 i-motif stem and loop mutants, respectively. (C) Table illustrating sequences of the i-motif mutants used in the binding study.}
\end{figure}
Figure 4.13. Binding Interaction Study of BCL2 i-Motif DNA Mutants With hnRNP LL RRM1 and RRM2. (A & B) Binding affinity of RRM1 and RRM2 to five of the BCL2 i-motifs with specific modification at the C-rich region and four of the modified BCL2 i-motif mutants with specific modifications at loops flanking the C-rich region, respectively. lane 17, BCL2-i_35T and 10 equivalents of RRM1; lane 18, BCL2-i_5T and 10 equivalent of RRM2. (C) Histogram representing percentage formation of RRM1, RRM2 and i-motif mutant complexes.

4.2.5. FRET between i-Motif and RRM1.

The interaction of RRM1s and the i-motif was further investigated by studying FRET between an amino acid donor in the RRM1s and a nucleotide acceptor into i-motif. Several constructs were made using site-directed mutagenesis, at different positions of the RRM1 sequence (Figure 4.14B), to convert native amino acid residues into amber codons. Plasmids containing an amber codon were subjected to in vitro protein synthesis, with modified tRNA charged with a 6-cyanotryptophan (Figure 4.14A), which afforded the modified constructs with incorporation of cyanotryptophan, as a fluorescent donor molecule, in different positions of RRM1, including Y137, Y147 and Y104. Purified proteins were used in FRET analysis with i-motif modified at the L3 central loop (BCL2i-22C) with a cytidine analogue (Figure 4.14A) as an acceptor, and the distance
between donor and acceptor was calculated from a FRET efficiency measurement, as outlined in section 4.4.14.

![Figure 4.14](image)

**Figure 4.14.** FRET Experiment With Amino Acid Substitution at Three Different Positions of RRM1. (A) Donor and acceptor molecules. (B) The tyrosines residues in RRM1 were alternatively substituted by 6-cyanotryptophan in three different constructs.

RRM1-137-CTP was synthesized by employing the *in vitro* protein synthesis method, using tRNA_{CUA} charged with 6-cyanotryptophan (CTP) and a plasmid containing RRM1 coding region with an amber codon at tyrosine 137 (Y137). Five µM RRM1-137-CTP was excited at 310 nm (excitation slit width = 10 nm) and titrated with BCL2i-22C up to 200 nM DNA, ensuring maximum binding. Emission (emission slit width = 10 nm) was monitored from 325 nm to 550 nm (Figure 4.15A). A similar experiment with 20 µM RRM1-wt and 200 nM BCL2i-22C was performed with no observable increment of emission peak of the acceptor at 440 nm (Figure 4.15B). Similarly RRM1-147-CTP and RRM1-104-CTP were synthesized by *in vitro* protein synthesis with plasmids containing an amber codon at tyrosine 147 (Y147) and tyrosine 104 (Y104), respectively. FRET
experiments with these proteins and BCL2i-22C showed significant energy transfer between donor and acceptor only when the donor was placed at Y104 (Figure 4.16B).

**Figure 4.15:** FRET Between RRM1-137-CTP and BCL2i-22C at 310 nm Excitation. (A) RRM1-137-CTP. (B) Wild type protein highlighted in purple line shows no significant FRET.

The interaction between RRM1 and the i-motif was simulated using docking tools affording numerous possible binding solutions. Exemplified in Figure 4.17A, a model oligonucleotide (CCCCA) was used as the substrate for RRM1. The distance between a donor (amino acid analogue) and acceptors (nucleotide substituents) can be measured
based on the location information available for the acceptors. In Figure 4.17B, three of the best fitting RRM1-oligomer binding solutions are reported, assuming that the donor is Y104 and that the acceptors are located on both termini of the DNA. Comparison of the simulation derived distances with the experimentally determined distance between the donor and acceptor supports the potential of this method to afford reasonably accurate molecular prediction of binding. A more realistic and detailed analysis is possible when the FRET experiments are carried out with donors at different locations resulting from

Figure 4.16: FRET Between (A) RRM1-147-CTP, (B) RRM1-104-CTP and BCL2-22C at 310 nm Excitation.
position-based amino acid substitutions (Figure 4.14), under a variety of experimental conditions. The FRET efficiency between the donor at Y137 and Y104 and acceptor in the i-motif was calculated following the method described in the experimental section. The FRET efficiency between the donor and acceptor from the RRM1-137-CTP / i-motif pair was 0.30±0.03. Similarly, the FRET efficiency between the donor and acceptor from RRM1-104-CTP / i-motif pair was 0.12±0.01. From the FRET efficiency, the distance between donor and acceptor was also calculated (36.0±0.7 Å when the donor was at Y104, and 29.8±0.6 Å when the donor was placed at Y137) and the information was used for the simulation of i-motif and RRM1 interaction an unambiguous distance restraint, using the Haddock web-server modeling tool (Figure 4.18).\textsuperscript{114-116}

Figure 4.17. Simulation of the Interaction Between Oligonucleotide CCCC A and RRM1. (A) Simulation of possible RRM-ssDNA interaction. (B) Locations of the amino acid donor at Y104 and nucleotide acceptors placed at the 5′ (A, B and C) or 3′-termini (a, b or c) of the DNA in three different models.
4.3. Discussion

In this study BCL2 i-motif DNA has been shown to interact with at least two of the individual domains of hnRNP LL (Figure 4.4). The binding interaction also imposed considerable change in the structure of the BCL2 i-motif (Figures 4.7 and 4.10). As shown in Figure 4.4 domains 1 and 2 bind to i-motif DNA with higher affinity, in comparison with RRM3-4. A comparative study (Figure 4.4C and D) suggests that RRM2 may have a higher affinity of binding to the i-motif than RRM1, analogous to domains 2 and 1 of hnRNP L, respectively. Interestingly, domain 2 of hnRNP L has also been reported to have higher binding affinity toward specific RNA substrates in previous studies.117 In a combination of the i-motif DNA mixed with calf thymus DNA (Figure 4.5A and B), RRM1 and RRM2 showed a moderately higher binding affinity for the i-motif DNA. Both domains bind selectively to the i-motif when incubated with an equimolar mixture of i-motif and random DNA, suggesting the existence of a potential recognition motif within the oligonucleotide sequence of the i-motif.
In previous studies, it was proposed that binding of the i-motif with hnRNP LL leads to a significant structural change in the i-motif,\textsuperscript{113} which in turn initiates the process of downstream transcription of the gene. The uniform banding pattern of the i-motif DNA using bromine footprinting analysis at pH 7.5 (lanes 2 and 5 in Figure 4.8) suggests that the DNA is already in a less structured form, supporting the previous finding that at \textit{BCL2} i-motif DNA exhibits the structure at lower pH. The transition pH for the oligonucleotide to undergo a structural change from the complex i-motif to a simpler form has been reported to be pH 6.6.\textsuperscript{113} Incubation of the i-motif with either RRM1 or RRM2 (lanes 3 and 4 in Figure 4.8) at pH 7.5 did not change the structure further. In cells the i-motif is found in the promoter region of the oncogene, which encodes the protein Bcl-2. In a cellular environment the promoter may retain the complex i-motif structure in
a locally favorable acidic environment. The C-rich region of the promoter has been also reported to bind to transcription factors, undergoing structural changes and facilitating transcription. In our study, when the i-motif was subjected to bromine footprinting assay at pH 6.6 it showed a non-continuous cleavage band pattern in the denaturing gel (Figure 4.7A, lane 2), suggesting that most of the cytidines in the oligonucleotide sequence are taking part in forming an i-motif structure as confirmed in previous studies. Intriguingly, when incubated with RRM1 and RRM2, the cytidines were shown to be more available for bromination and subsequent cleavage by pyridine, which suggests that the binding resulted in a structural change of the DNA, from a more complex i-motif to a simpler linearized DNA (Figure 4.7A, lanes 3 and 4). In a cellular environment, this phenomenon may lead to the initiation of downstream transcriptional processes (Figure 4.19).

![Proposed Mechanism of RRM1 and RRM2 Interaction With i-motif DNA and Subsequent Unfolding of the Complex Structure.](image)

**Figure 4.19.** Proposed Mechanism of RRM1 and RRM2 Interaction With i-motif DNA and Subsequent Unfolding of the Complex Structure. Both the domains of hnRNP LL were postulated to interact with the lateral loops L1 and L2 of i-motif to initiate relaxation of the complex stem–loop structure of the oligonucleotide.

The structural change of the i-motif was studied by obtaining CD spectra of the combination of oligonucleotide and the domains of hnRNP LL at pH 6.6. As evident in
both Figures 4.10 and 4.11, the structure of the i-motif changes greatly as a result of the interaction with RRM1 and RRM2. In both Figures (4.10 and 4.11), the observed decrease of the characteristic i-motif peak at 286 nm with increasing concentration of protein suggested that the i-motif structure may have been gradually converted to a linear DNA upon binding. The increasing area within the negative peak at 210 nm is characteristic of protein domains, which was consistent with the increase in protein concentration in subsequent experimental steps. In Figure 4.10B, the structural change of the i-motif is more prominent, which is represented by the blue line (5 molar equivalents of RRM2), indicating the strong binding interaction of RRM2 with the i-motif DNA, ultimately resulting in a shift of the 286 nm peak to 270 nm.

A thorough binding assay employing modifications of the i-motif DNA (Figure 4.12C) with the RRM5s revealed that the specific interaction of hnRNP LL domains with the i-motif is more dependent on the loop sequences and less likely depends on the specific structure of the DNA (Figure 4.13B). The complex C-rich region of i-motif may not interact with the domains and in fact, subtle changes in the i-motif structures (Figure 4.12A) do not affect binding. In accordance with the previous studies, it was confirmed that the domains bind to the loops of the i-motif DNA and may have little interaction with the C-rich region (Figure 4.19).

To further extend our knowledge of i-motif and RRM1 interaction, the FRET technique has been utilized with i-motif DNA and the domains of hnRNP LL. Based on the binding interaction study, a nucleotide in the region of the central loop (19 to 24) was replaced by a synthetic nucleotide analogue, which can serve as an energy acceptor in the FRET experiments. Replacement of the cytidine nucleotide with a fluorescent analogue
of cytidine (Figure 4.14) afforded BCL2i-22C. Similarly, replacing a tyrosine (Y137, Y147 and Y104) by a synthetic unnatural amino acid analogue provided the FRET donor. FRET efficiencies between the donors at positions 137 and 104 and the acceptor at a fixed position in BCL2i-22C were calculated using the method described in section 4.4.15, which also afforded the calculation of the distances between Y137 or Y104 and the cytidine nucleotide of the BCL2 i-motif, while bound to the protein. The FRET efficiency between the donor at position Y147 and BCL2i-22C with the acceptor at C22 was exceptionally low (<10%), hence not used for further distance calculation to avoid a higher order of error. The FRET efficiency and the distance calculations were performed using a simplified method without involving the corrections for the donor and acceptor environments, which will be subjected to further speculations involving additional experiments. The distances involving Y137 and Y104 were used in the Haddock webserver based simulations\textsuperscript{114-116} to gain more insight into RRM1–BCL2 i-motif binding interaction, as illustrated in Figure 4.20.
Figure 4.20. Modeling of RRM1 and i-Motif Interaction With or Without Using FRET Derived Distance Information. (A) Rigid body docking without using an unambiguous restraint. (B) Flexible docking of RRM1 and the i-motif DNA, which was based on modeling involving a flexible DNA backbone and experiment derived distances between specific amino acids and the nucleotide, C\textsubscript{22} as unambiguous restraints.

4.4. Experimental

4.4.1. End-labeling of Oligonucleotides

*BCL2* i-motif was [5'-\textsuperscript{32}P]-end labeled with γ-\textsuperscript{32}P ATP + T4 polynucleotide kinase enzyme. Ten pmol of DNA was 5'-\textsuperscript{32}P end labeled by incubation with 20 units of T4 polynucleotide kinase and 0.06 mCi [γ-\textsuperscript{32}P]ATP (specific activity 6000 Ci (222 TBq)/mmol) in 50 μL (total volume) of 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl\textsubscript{2} and 5 mM DTT. The reaction mixture was incubated at 37 °C for 1 h followed by purification of DNA by 16% polyacrylamide gel electrophoresis at 1800 V for 2.5 h.
4.4.2. Maxam-Gilbert Sequencing Reaction\textsuperscript{65}

Ten \( \mu L \) of labeled DNA solution (~50000 cpm) was treated with 25 \( \mu L \) of formic acid and incubated at 25 °C for 4-5 min. The reaction was stopped by treatment with 200 \( \mu L \) of 0.3 M sodium acetate, pH 7.0 mixed with 0.1 mM EDTA and 25 \( \mu g/mL \) tRNA. The resulting solution was containing 700 \( \mu L \) of ethanol and the DNA was precipitated. The DNA pellet was purified by ethanol precipitation and resuspended in 75 \( \mu L \) of 10% piperidine. The reaction mixture was incubated at 90 °C for 30 minutes, cooled down and the supernatant was removed under diminished pressure. The DNA pellet was washed and mixed with denaturing loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue and 1% xylene cyanol, then heated at 90 °C for 10 min and stored in −20 °C.

4.4.3. Electrophoretic Mobility Shift Assay

The \textit{BCL2} i-motif and each of the mutants mentioned in Table 1 were \([5'-32P]\)-end labeled as noted above. These constructs were prepared in 50 mM Tris buffer with 100 mM NaCl at pH 7.0. One \( \mu L \) (10000 cpm) of each of those DNAs were mixed with 10 \( \mu M \) non-radioactive oligonucleotide counterparts. Ten pmol of each of those DNAs were then incubated with 2.5, 5, 7.5 on 10 pmol of RRM1 or RRM2 for 15 min at room temperature. The DNA–protein mixtures were subjected to 12% non denaturing gel electrophoresis at 80 V for 1.5 h. The resulting bands were visualized using a phosphorimager.
4.4.4. Bromine Footprinting Assay

The bromine footprinting assay protocol was adapted from the previous procedure.\textsuperscript{106} BCL2 i-motif was [5'-\textsuperscript{32}P]-end labeled as described previously. Ten pmol of the purified DNA was incubated with bromine formed \textit{in situ} by mixing a 50 mM solution of KBr and KHSO\textsubscript{5} for 20 min at room temperature in a total volume of 20 µL. The reaction was terminated by the addition of 200 µL of 0.3 M sodium acetate containing 10 mg/mL calf thymus DNA and 700 µL of cold ethanol. The reaction mixture was incubated in ~80 °C for 20 min and the DNA pellet was purified by additional ethanol purification steps. The DNA pellet was resuspended in 70 µL of 10% piperidine. The reaction mixture was incubated at 90 °C for 30 min, cooled and the supernatant was removed under diminished pressure. The DNA pellet was washed with water and mixed with denaturing loading buffer containing 80% formamide, 2 mM EDTA, 1% bromophenol blue and 1% xylene cyanol, then heated at 90 °C for 10 min and resolved in a denaturing polyacrylamide gel.

4.4.5. Circular Dichroism

CD experiments were performed based on the protocol described in literature.\textsuperscript{106} The spectral data was collected using a Jasco-810 spectropolarimeter and a quartz cell of 1.0 mm path length. Spectra were obtained at a scanning speed of 100 nm/min with a response time of 1 sec, over a wavelength range of 200-350 nm. For each scan, the data was recorded six times, averaged, smoothed and baseline corrected to eliminate the signal contribution from buffer. The oligonucleotides were dissolved in 50 mM Tris buffer (pH 6.6 and 7.0) containing 100 mM NaCl at a concentration of 5 µM. RRM1 and RRM2
were prepared in same buffer at 50 µM concentration. For binding interaction studies, CD spectra were obtained after incubating DNA and protein at room temperature for 15 min.

4.4.6. Mutagenesis and Subcloning

Three PCR (Polymerase Chain Reaction) site-directed mutagenesis procedure were carried out using NEB Q5 Mutagenesis kit. The forward and reverse primers for each PCR mutagenesis are listed in the Appendix. NEB base changer web-tool was used to design the primers, in order to incorporate an amber codon (TAG) at either Y137 or Y104 and Y147. PCR reactions were performed in 25 µL reaction mixtures, following the procedure described in the kit manual. Each reaction mixture contained 25 ng wild-type template RRM1, encoded in a pET 28a vector, 125 ng of forward and reverse primers, 10 nmol of dNTPs, 2.5 units of DNA polymerase in 35 mM Tris-HCl at pH 8.0 containing 12 mM potassium acetate, 5mM DTT and 0.05% Triton X-100 in 0.05 mM EDTA. A combination of the forward and reverse primers, 137-Forward and 137-Reverse or 104-Forward and 104-Reverse were used for the synthesis of RRM1-137TAG and RRM1-104TAG, respectively, whereas, 147-Forward and 147-Reverse were used for the synthesis of RRM1-147TAG. The products were ligated with the Q5 ligase master mix and transformed in DH5α E. coli cells. Purified plasmids were verified by sequencing. The product plasmids were the RRM1 constructs with a TAG codon at position 137 or 104 and 147.
4.4.7. Competent Cell Preparation.

A stock of DH5α competent cells was made for transformation, following the Invitrogen competent cell preparation protocol. The preparation was carefully done at −4 °C in a sterile environment. A 250 mL LB culture produced a stock of 21.4 mL cells in 97 tubes of 220 uL aliquots. The transformation efficiency of the cells was 2.73X10^7 when checked with pUC19 vectors.

4.4.8. NVOC protected aminoacyl Dinucleotide Preparation.

Crude aminoacylated pdCpA samples were dissolved in DMSO. The concentration of the resulting solution was checked by measuring the absorbance of the dinucleotide, at 260 nm wavelength and was found to be 96 ABS (absorbance unit). The pdCpA solutions were used for tRNA ligation to a final concentration of 5.0 ABS.

4.4.9. Transcription of 74-Nucleotide tRNA and Purification

The plasmid pYRNA8, which encodes a 74-nucleotide tRNA (tRNA-C_OH) was transformed into DH5α cells and a single colony was picked for overnight growth in 500 mL LB media supplemented with 100 µg/mL ampicillin. The harvested cells were used for large-scale plasmid extraction following the Promega maxiprep-kit protocol. The Plasmid concentration was measured by checking the absorbance at 260 nm and a stock of 1 µg/mL solution was prepared by dissolving the plasmid in water.
4.4.10. Digestion of pYRNA8 Plasmid with FokI

The pYRNA8 was completely linearized for the subsequent activity of the T7 RNA polymerase. The plasmid was digested using FokI restriction endonuclease. The linear DNA was separated from protein impurities and purified by Phenol–Chloroform extraction followed by ethanol precipitation. The digested product was run in a 1% agarose gel along with undigested plasmid as a control. The fastest migrating band was collected and purified. The DNA fragment containing 74-nucleotide tRNA, being expressed under a T7 promoter, was subjected to in vitro transcription. The conventional ampliscribe T7 transcription protocol was followed and based on previous data; a high yield of 74-nucleotide RNA was obtained with an additional use of 500 µg GMP in the transcription buffer.

4.4.11. Purification of tRNA by DEAE Sephadex Chromatography

After transcription, the reaction mixture was precipitated by the addition of 40 µL of 3M NaOAc at pH 5.0 and 1.3 mL of cold EtOH, then washed with 70% EtOH. The product was air dried and dissolved in 300 µL of 0.1 M NaOAc, pH 5.0. The solution was loaded onto 800 µL of Sephadex resin, equilibrated with the same buffer mentioned above and eluted with a step gradient of NaCl [0-0.9 M]. The fractions were purified and analyzed by electrophoresis on an 8% PAA–Urea [polyacrylamide–urea] gel at pH 8.4 (TBE buffer) for 1h at 100 V. The tRNA was used in a ligation reaction with aminoacylated pdCpA in the presence of ATP and T4 RNA ligase.
4.4.12. Ligation of tRNA-C\text{OH} with Aminoacyl-pdCpA and NVOC Deprotection

Suppressor tRNA aminoacylation was carried out in 100 mL (total volume) of 100 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, containing 2.0 mM ATP, 15 mM MgCl\text{2}, 100 μg of suppressor tRNA-C\text{OH}, 5.0 A\text{260} units of NVOC-protected 6-cyanotryptophanyl-pdCpA, 15% DMSO and 200 units of T4 RNA ligase. After incubation at 37 °C for 1 hour, the reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixture was incubated at −20 °C for 30 minutes, then centrifuged at 15,000 × g at 4 °C for 30 minutes. The supernatant was carefully decanted and the tRNA pellet was washed with 100 μL of 70% ethanol and dissolved in 30 μL of RNase free H\text{2}O. The NVOC-protected 6-cyanotryptophanyl-tRNA was cooled to 2 °C and irradiated with a 500 W mercury-xenon lamp for 5 minutes.

4.4.13. S-30 Preparation

S-30 systems from \textit{E. coli} BL-21 (DE-3) containing \textit{rrnB} genes were prepared by procedure described in literature.\textsuperscript{118,119} Cells were grown at 31 °C in LB medium, supplemented with ampicillin (100μg/L), until the optical density at 600 nm was 1.0. The cell solution was then diluted 10 times with LB medium and IPTG (500 μg) and growth was continued until the optical density was 2.0. The cells were harvested and lysed by egg lysozyme (150 μg/g of cells). Finally, the S-30 extract was dialyzed, aliquoted in 100 μL micro-centrifuge tubes and stored at -80 °C.

Circular DNA plasmid containing the gene of interest can be transcribed and translated in one single reaction as long as the T7 polymerase is present. The transcription and translation reactions are done in one tube, i.e. termed as a “coupled” system. The procedure is also referred to as “*in vitro* translation”. Reactions were carried out following a standard procedure developed in the Hecht Laboratory in 10 µL reaction mixtures, with 600 ng plasmid containing the gene for the relevant protein, in 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 µg/µL aminoacyl tRNA, 3.5% polyethylene glycol (PEG)-6000, 20 µg/µL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 mM mixture of amino acids, 0.1-0.4 µl/µL of S-30 preparation and 100 µM (0.5 µCi/µl) of ³⁵S-methionine.

In the suppression analysis with a plasmid containing a TAG codon, aminoacylated tRNAs were added to the reaction mixture up to a concentration of 0.3 µg/µL. Reactions were incubated at 37 °C for one hour. Purification of the protein was done following the Strep-Tactin manufacturer’s protocol (Iba-LifeSciences). Reaction yields were analyzed by visualizing the protein band in a 15% Tris-glycine SDS-PAGE gel and quantification of protein was done by imagephosphor software. *In vivo* protein expression was done by adding IPTG to an *E. coli* BL-21 (DE-3) cell culture after 3 hours, containing RRM1, RRM2 or RRM3-4 plasmids. The cultures were incubated overnight at 37 °C, followed by the addition of rifampicin (1 µg/mL) and incubation for another 12-16 hours before lysing the cells by sonication. The separation of proteins from the cellular debris was performed by centrifugation at 4000 rpm. The supernatant was
filtered and loaded directly on a Strep-Tactin column. The protein was eluted with 50 mM PBS buffer.

4.4.15. FRET Experiment

The FRET efficiency between donor and acceptor was measured experimentally by gradual addition of DNA to 5 μM RRM1 solution at pH 4.5. The protein was incubated with the DNA for 5 min and then excited at 310 nm. Emission was measured immediately in the range of 325-550 nm. The emission profiles of the donors (RRM1-137-CTP, RRM1-104-CTP) and the donor–acceptor complexes were normalized, integrated and fitted using the equation, \[ E = \frac{I_A}{I_A + I_D} \], where \( E \) is the FRET efficiency; \( I_A \) and \( I_D \) are the integrated intensities of the donor in unbound and bound forms, respectively. The distance between donor and acceptor was calculated by fitting FRET efficiency in the equation, \[ R = R_0 \times ((1-E)/E)^{1/6} \], where \( R_0 \) is the Förster radius. \( R_0 \) value was calculated by Poulami Talukder to be 26 Å using the method described in ‘Introduction to Fluoroscence’ by David Jamesons.\(^{120}\)

4.4.16. Graphical Modeling and Simulation of DNA–Protein Interaction

RRM1 was modeled based on homology modeling using Modeller.\(^{121,122}\) The fluorescence data was individually baseline corrected, normalized, integrated and used for FRET efficiency calculations using statistical data processing software, Origin 8. The FRET efficiency and distances between donors and acceptor were calculated using the method described in the previous section. A pdb file for the 39-nucleotide long i-motif was generated using the Make-NA algorithm.\(^{123}\) The DNA binding residues in RRM1
were found using DP-bind web-tool. The predicted active residues were R105, G106, F134, K135, R136, Q137, Q165, F168, N170, Y171. Based on the current study and the previously reported results, the active residues of i-motif were found to be in loop L1 and L3 (lateral loops). The binding pockets on RRM1 were scanned by Patchdock web-tool for favorable binding interactions, which is followed by the rigid body docking, using Hex-docking algorithm. The data was confirmed by the Haddock docking algorithm (Figure 4.20 A), using active residues as ‘ambiguous-binding restraints’. Furthermore, the dynamic docking was performed using Haddock expert interface (~24 hours processing time for each pair), considering flexible regions of protein and DNA, solvent factors, polar-nonpolar interactions, energy minimization, hydrogen bonding and backbone conformational restraints. The FRET experiment derived data was used as unambiguous restraints for dynamic modeling (Figure 4.20B). The represented data was the best model from a library of over 1000 virtual modeling results. The molecules were visualized and virtual mutations were done using PyMol software.
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APPENDIX A

DNA AND PROTEIN SEQUENCES OF THE RRM CONSTRUCTS
A.1. hnRNP LL isoform_1 (NP_612403)

1 msssssspre tyedreyes qakrlkteeg eidysaege nrreatprgg gdgggggrsf
61 sqpeaggshh kvsvspvvh rglcesvvea dlvealekfg ticyvmmmpf krqalvefen
121 idsakecvtf aadeepvyiag qqaffnysts kritrpgnv ndpsqgnkvll lsiqnpypipi
181 tvdlytvcn pvkgvqrvivi fkrnqigamv efesvlcaqk akaalngadi yagcctlkie
241 yarptrlnvii rndonswydt kpylgrrdrg kgrqrqailg ehpssfrhdg ygshgplpl
301 psrymgsrd tpelvayplp qasssymhgg npsgs vvmvs glhqlkmncs rvfnlfclyg
361 niekvkfmkt ipgtalvemg deyaveravt hlnnvklfgk rlnvcvskqh svvpsqifel
421 edgtssykdf amsknrfts agqaskniiq ppescvihyn vplcvteef tklcndhevl
481 tfikykvfd aksaktlsqgl lewecktdav ealtainhyq irvpngsncy tklcfstss
541 hl

Red amino acids indicate RRM1
Blue amino acids indicate RRM2
Green amino acids indicate RRM3
Purple amino acids indicate RRM4

A.2. Constructs used in this study

Each of the constructs had an NcoI site (CCATGG) at the 5’-end, with an embedded transcription start site (ATG) followed by a fused glycine sequence (GGT) from the original reading frame of the RRM domains. The constructs are attached to a 24 nucleotide long Strep-tag sequence (Yellow bases) followed by a stop codon (TAA) at the 3’-end.

A.2.1. RRM1 gene sequence

5’--CCATG

GGTCGTCTTTTCTCTCAGCGGAAACGGTGGTTCTCACCAACAAAGTTCTGTTTCTCC
GGTTGGTCACGTCGGTTGCTGCAATCTGGTGGGAAGCGGACCTTGGTTGAAGCGC
TGGAAAAATTCGGTACCATTCTGCTACGTGGCATGATGATGCCGTTCAACGTCAGGCGT
GGTTGAAATTCGGAGGACATACGTACTCGGCAGAAATGCGTTACCTTCCGGCGGACGAAC
GGTTTACATCGCGGGTCAGCAGCGGTCTTCAACTACTCTCCCTTAAACGTATCACCC
A.2.2. RRM1 amino acid sequence (13 kDa)

MGRSFSEQPEAGGSHHKVSVSPVVHRGLCESVVEADLVEALEKFGTICYVMMPFKRQA

LVEFENIDSAKECVTFAADEPVYIAQQAFFNYSTSKRITRPGNTDDPSGG

WSHPQFEK*

A.2.3. RRM1 coding region in pET 28a vector

3   ATG GGT CGT TCT TTC TCT CAG CCG GAA GCG GGT GGT TCT CAC CAC   47
 1   Met Gly Arg Ser Phe Ser Gln Pro Glu Ala Gly Gly Ser His His
 15
 48  AAA GTT TCT GGT TCT CGG GTT GTT CAC GTT CGT GGT CTG TGC GAA   92
 16  Lys Val Ser Val Arg Val His Arg Val Val Arg Gly Leu Cys Glu
 30
 92  TCT GTT GAA GCG GAC CTG GTT GAA GCG CTG GAA AAA TTC GGT   137
138  Ser Val Val Glu Ala Asp Leu Val Glu Ala Leu Glu Lys Phe Gly
 45
182  ACC ATC TGC TAC GTT ATG ATG ATG CCG TTC AAA CGT CAG GCG CTG  182
196  Thr Ile Cys Tyr Val Met Met Met Pro Phe Lys Arg Glu Ala Leu
 60
227  Val GAA TTC GAA AAC ATC GAC TCT GCG AAA GAA TGC GTT ACC TTC
227  Val Glu Phe Glu Asn Ile Asp Ser Ala Lys Glu Cys Val Thr Phe
 75
272  GCG GCG GAC GAA CCG GTT TAC ATC GCG GGT CAG CAC GCG TTC TTC
272  Ala Ala Asp Glu CCG Val Tyr Ile Ala Gly Glu Glu Ala Phe
 90
317  AAC TAC TCT ACC TCT AAA CGT ATC ACC CGT CCG GGT AAC ACC GAC  317
317  Asn Tyr Ser Thr Ser Lys Arg Ile Thr Arg Pro Gly Asn Thr Asp
105
362  GAC CGG TCT GTG GGT TGG TCT CAC CGG CAG TCC GAA AAA TAA ---  362
362  Asp Pro Ser Gly Gly Trp Ser His Pro Gln Phe Glu Lys End XXX
119

A.2.4. RRM2 gene sequence

5’ --CCATG

GGTAACACCGACGACCGTCCTGGTGTAACAAAGTTCTGTGTCTATCCAGAACCC

GCTGTACCCGATCACCCTTGACGTTCTGGTACACCGTGTGCAACCCGGTGGTAAGTTC

AGCGTATCTGTTATCTTTAAACGTAACCGTGATCCAGCTGAGTGGTAGTAACGCGT

126
A.2.5. RRM2 amino acid sequence (16 kDa)

**2.5. RRM2 amino acid sequence (16 kDa)**

MGNTDDPSGNKVLLLSIQNPLYPTDVLYTVCNPVGKVQIVIFKRNGIQAMVEFES

VLCAQKAKAALNGAIDIYACCTLKIEYARPRTLNVIRNDNSWDYTPYLGRRDREGK

WSHPQFEK*

A.2.6. RRM2 coding region in pET 28a vector.

```
3    ATG GGT AAC ACC GAC CCG TCT GGT GGT AAC AAA GTT CTG CTG   47
1     Met Gly Asn Thr Asp Asp Pro Ser Gly Gly Asn Lys Val Leu Leu   15
48   CTG TCT ATC CAG AAC CCG CTG TAC CCG ATC ACC GTT GAC GTT CTG   92
16    Leu Ser Ile Gln Asn Pro Leu Tyr Pro Ile Thr Val Asp Val Leu   30
93   TAC ACC GTT TGC AAC CCG GTT GGT AAC AAA GTT CAG CGT ATC GTT ATC 137
31    Tyr Thr Val Cys Asn Pro Val Gly Lys Val Gln Arg Ile Val Ile   45
138  TTC AAA CGT AAC GGT ATC CAG GCG ATG GTT GAA TTC GAA TCT GTT 182
46    Phe Lys Arg Asn Gly Ile Gln Ala Met Val Glu Phe Glu Ser Val   60
183  CTG TGC GCG CAG AAA GCG AAA GCG CCG CTG AAC GGT GCG GAC ATC 227
61    Leu Cys Ala Gln Lys Ala Lys Ala Ala Leu Asn Gly Ala Asp Ile   75
228  TAC GCC GGT TGC GCG ACC CTG AAA ATC GAA TAC GCC CGT CCG ACC 272
76    Tyr Ala Gly Cys Thr Leu Lys Ala Lys Ala Leu Asn Gly Ala Asp Ile   90
273  CTG CTG AAC GGT ATC CGT AAC GAC AAC GAC TCT TGG GAC TAC ACC 317
91    Arg Leu Asn Val Ile Asp Asp Asn Asp Ser Trp Asp Tyr Thr   105
318  AAA CCG TAC CTG GGT GGT GCC GGT GAA GGT GGG GTG TCT CAC 362
106   Lys Pro Tyr Leu Gly Arg Arg Asp Arg Gly Lys Gly Trp Ser His   120
363  CCG CAG TTC GAA AAA TAA --- 383
121   Pro Gln Phe Glu Lys End XXX   126
```

A.2.7. RRM3-4 gene sequence

5' --**CCATG**
A.2.8. RRM3-4 amino acid sequence (24 kDa)

MGGSNSGSMVSMGLHQLKMNCRVSFLFCFLYGNIEKVKFMTTPGTALVEMGDEYAVE
RAVTHLNNVKLFKRNVCVSHSVPSQIELEDGTSYKDFAMSKNRFAGQAS
KIIPPSCVLHYNYVPLCVTEEFTKLCNDHEVLTFIKYKVFDAKPSAKTLSGLLEWE
CKTDAVEALTALNHYQIRVPNGSNPYTLKCFSTSSHL
WSHPQFEK*

A.2.9. RRM3-4 coding region in pET 28a vector.

1  47  ATG GTA AAC CCN TCT GTG GTA ATG GTA TCT GTG GTA
15  48  Met Gly Gly Asn Pro Ser Gly Ser Val Val Met Val Ser Gly Leu
30  16  His Gln Leu Lys Met Asn Cys Ser Arg Val Phe Asn Leu Phe Cys
129

93  CTG TAC GGT AAC ATC GAA AAA GTT AAA TTC ATG AAA ACC ATC CCG  137
31  Leu Tyr Gly Asn Ile Glu Lys Val Lys Phe Met Lys Thr Ile Pro  45

138  GGT ACC GCG CTG GTT GAA ATG GGT GAC GAA TAC GCG GTT GAA CTG  182
46  Gly Thr Ala Leu Val Glu Met Gly Asp Glu Tyr Ala Val Glu Arg  60

183  GGT ACC CAC CTG AAC AAC GTT AAA CTG TTC GGT AAA CGT CTG  227
61  Ala Val Thr His Leu Asn Asn Leu Val Phe Gly Lys Arg Leu  75

228  AAC GTT TGC GTT CAC TCT AAA CAG CAC TCT GTT GTT CCG TCT CAG ATC  272
76  Asn Val Cys Val Ser Lys Gln His Ser Val Val Pro Ser Gln Ile  90

273  TTC GAA CTG GAA GAC GGT ACC TCT TCT TAC AAA CAG CAC TCT GCG GTT  317
91  Phe Glu Leu Gly Asp Gly Thr Ser Ser Tyr Lys Asp Phe Ala Met  105

318  TCT AAA AAC AAC CTG TCT ACC TCT GCG GGT GAC GGT TCT AAC GAC  362
106  Ser Lys Asn Asn Arg Leu Thr Thr Gln Gly Asp Gly TCT AAC GAC  120

363  ATC ATC CAG CCG CCG TCT TGC GTT CAC TAC CAC TAC AAC GTC CCG  407
121  Ile Ile Gln Pro Pro Ser Cys Val Leu His Tyr Gln Asp Val Pro  135

408  CTG TGC GTT ACC GAA GAA ACC TTC ACC AAA CTG TGC AAC GAC CAC  452
136  Leu Cys Val Thr Glu Glu Thr Phe Thr Lys Leu Cys Asn Asp His  150

453  GAA GGT CTG ACC TTC ATC AAA TAC AAA GTT TTC GAC GCG AAA CTG  497
151  Glu Val Leu Thr Phe Ile Lys Tyr Val Phe Asp Ala Lys Pro  165

498  TCT GCG AAA ACC CTG TCT GTG CTT CTC AAA GAA TGC GAA TGG GAA TGC AAA ACC  542
166  Ser Ala Lys Thr Leu Ser Gly Leu Leu Glu Trp Glu Cys Lys Thr  180

543  GAC GCG GTT GAA GCG CTG ACC GCG CTG AAC CAC TAC CAG ATC CTG  587
181  Asp Ala Val Glu Ala Leu Thr Ala Leu Asn His Tyr Gln Ile Arg  195

588  GTT CCG AAC GGT TCT AAC CCG TAC ACC CTG AAA CTG TGC TTC TCT  632
196  Val Pro Asn Gly Ser Asn Pro Tyr Thr Leu Lys Leu Cys Phe Ser  210

633  ACC TCT TCT CAC CTG TGG TCT CAC CCG CAG TTC GAA AAA TAA ---  677
211  Thr Ser Ser His Leu Trp Ser His Pro Gln Phe Glu Lys End XXX  224
APPENDIX B

LIST OF PRIMERS FOR RRM1 MUTAGENESIS
### B.1. RRM1 mutagenesis primers

<table>
<thead>
<tr>
<th>mutagenesis primer</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>137-Forward</td>
<td>AACCGGTTTAgATCGCGGGTC</td>
</tr>
<tr>
<td>137-Reverse</td>
<td>CGTCCGCCGCGGAAGGTAA</td>
</tr>
<tr>
<td>104-Forward</td>
<td>CCATCTGCTAgGTTATGATGATG</td>
</tr>
<tr>
<td>104-Reverse</td>
<td>TACCGAATTTTTCCAGCG</td>
</tr>
<tr>
<td>147-Forward</td>
<td>TCTTCAACTAgTCTACCTCTAAACGTATCACCCG</td>
</tr>
<tr>
<td>147-Reverse</td>
<td>ACGCCTGCTGACCCGCGA</td>
</tr>
</tbody>
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