MASKING DNA SINGLE-STRAND BREAK (SSB): A NEW APPROACH TO CANCER TREATMENT

By

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I. Introduction

1.1 DNA damage and cancer

Cancer is a multifaceted disease. It usually involves a fast replication rate and a potentially unlimited proliferation of cells. These advantages allow cancer cells to form malignant tumors and spread around the body by competing and invading nearby tissues (1). As a multifactorial genetic disease, the possible causes of cancer can come from various sources (2). Previous studies have shown that DNA damage increases the risk of cancer (3). The source of this damage can come from both endogenous and environmental factors. Endogenous damage includes hydrolytic and oxidative reactions, and environmental damage could come from UV radiation and chemicals present in the environment, such as free radicals (4). In short, DNA damage is an inevitable event.

Incomplete or inappropriate repair of DNA damage will accumulate mutations which could ruin the genome such as by deleting and shifting of sequences, translocation of chromosomes, and many other permanent changes of DNA (4). Some of the consequences of mutations include genetic instability and cell death (3).

Single-strand breaks (SSB) are the most common lesions that happen to DNA. It occurs at a rate of tens of thousands per cell per day, which is far more frequently than most other types of errors such as UV-induced pyrimidine dimmers and bulky base-pair adducts (5). There are variety of mechanisms that can give rise to SSB, such as from enzymatic intermediates of most repair pathways and normal cell metabolic processes (4). SSB creates a discontinuity in one of the DNA double
helixes that is usually characterized by a missing base or a breach in the phosphate backbone.

Persistent SSB and environmental stresses such as irradiation and oxidants can lead to extremely deleterious damage in chromosomes: double-strand breaks (DSB). The presence of DSB can collapse the replication fork during cell replication, which stops cell division (6). Therefore, DSB poses a major threat to genome integrity and cell survival.

Cells are equipped with checkpoint mechanisms to inspect DNA damage during cell cycle (see figure 1 and caption). Cell cycle consists of 4 primary stages, G1, S, G2 and M. Each stage has a checkpoint. These checkpoints are inspecting DNA damages at different stages during cell cycle. Cells have to pass all 4 stages of cell cycle in order to completely divide (7). If DNA damage is detected, the cell cycle will be stalled at the check point to prevent further transferring of errors. The cell will then engage in various DNA repair responses in order to counteract DNA damage to continue the cell cycle (4).
A base excision repair (BER) pathway can repair the non-bulky base modification (8). This type of repair mechanism usually gives rise to SSB during the repairing process (see the labels on figure 2). BER can start by removing the problematic base in order to make an abasic site (AP site) by glycosylase action, followed by incision at 5’ and 3’ end by the AP endonuclease that makes 3’-hydroxyl and 5’-deoxyribose phosphate (dRP) termini (9). Then 5’-dRP is removed and the correct base is inserted by polymerase β (Polyβ) activity. In the case that the AP site is modified and develops resistance to Polyβ, a strand of 2 to 8 nucleotides is inserted in the AP site in order to repair the lesion (10). Then flap endonuclease 1 (FEN1) cuts the newly inserted strand and 5’-dRP. The last step of BER is to seal the gaps by ligase (9). This process is summarized in figure 2.
Evidence shows that more proteins are involved in the BER pathway (11).

Poly (ADP ribose) polymerase (PARP) is one of these proteins that is thought to have a crucial function in sensing DNA damage and initiating BER (12). PARP sends a signal to the repair proteins, such as DNA polymerase, when SSB occurs. These proteins arrive at the damaged site after receiving the signal. PARP is a highly conserved nuclear enzyme present in multicellular organisms. It is activated by DNA damage. It binds the SSB as a homodimer. Once PARP is bound to the DNA lesion site, its catalytic activity is activated and enhanced about 500 fold, allowing it to covalently link the poly-(ADP-ribose), which is then synthesized from NAD⁺, to nuclear proteins. These proteins mostly bind to DNA and are involved in DNA metabolism and chromatin structures. One of the consequences of PARP’s enzymatic activity is the accumulation of negative charges at the breakage, which also causes these modified nuclear proteins to lose affinity to DNA (12). As illustrated in figure 2C, SSB will stay in the DNA when PARP is not present. The unrepaired SSB will then lead to replication fork collapse during replication, which can lead to severe consequences, such as DSB during replication, and eventually cell death (figure 2C, 2D) (8).
Figure 2. A general representation of base excision repair pathway. AP endonuclease makes an abasic site at the lesion, creating a SSB. PARP is activated by the damage and binds to 5′ dPR, and starts to accumulate negative charge. BER repair proteins are recruited and fix the site through two different pathways. A: the abasic site is recognized and fixed through the strand displacement mechanism. Ligase fills up the gap. B: Pol β attaches the correct base and ligase fills up the gap. C: when PARP is inhibited, SSB remains, and eventually compromises genome stability and causes cell death by apoptosis. D: SSB and replication fork. Orange circle: polymerase. In a normal cell, PARP recognizes SSB and initiates BER. When PARP is inhibited, the replication fork will collapse and the replication process will be stalled.
Cells also have strategies to target DSB, one of which is through the homologous recombination (HR) pathway. The HR pathway involves strand invasion and repair synthesis to exchange genetic materials with the neighboring strand (see figure 3). BRCA1 and BRCA2 are involved in this process (6). The error-prone DNA repair mechanism is commonly presented in cancerous cells (13). When the repair pathways are damaged, the defects allow the mistakes to remain and replicate, and give rise to cancerous cells (5). Defects in BRCA genes can cripple the HR process, causing persistent DSBs in the replication process (14). HR is deficient in BRCA mutation associated cancer. These particular types of cancer cells are extremely sensitive to PARP inhibition, since the malfunction HR can’t repair DSB induced by persistent SSB. Blocking PARP activity accumulates SSBs as well as DSBs. The unrepaired DSBs will eventually lead to apoptosis (see figure3). The damaged repair mechanism harbors tumorgenesis, but it is also a weakness. We could explore the
weaknesses related to defective repair mechanisms to develop a new approach to cancer treatment.

Figure 3. A schematic representation of repairing DSB using the HR pathway during replication. Orange circle: DNA polymerase. Blue filled oval: BRCA1. Blue filled oval: BRCA2.

1.2 Current therapeutic strategies

Different methods have been developed to treat cancer. Surgery, radiotherapy, and chemotherapy are among the most popular choices. When cancer is contained in one area, surgery is performed to physically remove the solid tumor (15). This method is also frequently combined with radiotherapy and chemotherapy.
Radiotherapy uses high-energy radiation such as X-ray, gamma ray, and charged particles to make various radiation-induced DNA lesions that cause cell death (15). Chemotherapy can use cytotoxic drugs in order to target cancer cells (6). These three different methods have been frequently used in cancer treatment (15).

Although existing chemotherapy and radiotherapy show plausible results in containing cancer cell growth and relieving cancer symptoms, they come with limitations. It is challenging for both methods to specifically target only the cancer cells. Radiotherapy aims to give a high dosage of irradiation to cancer region, and the lowest possible dosage to the surrounding normal tissues. Still, irreversible damage can be done to surrounding normal cells (16). Some strategies of chemotherapy involve attacking rapidly dividing cancer cells; but this can become problematic when natural rapid dividing cells, such as cells in bone marrow, digestive tract, and hair follicles, are sometimes misidentified as tumor cells (15). Depleting these important cells during cancer therapies leads to common side-effects such as myelosuppression, mucositis, and alopecia (15).

New therapeutic approaches with less toxic side-effects and higher efficacy that exploit tumors directly are desired. Employing synthetic lethality in therapeutics can be one of the promising new methods.

1.3 The principle of synthetic lethality

Synthetic lethality describes a phenomenon where a mutation will not be lethal by itself, but would become deadly when combined with another mutation in
the same cell (17). This combination could cause cell death, as illustrated in figure 4 (18). We can use the same principle to exploit defective DNA repair in cancer cell to trigger apoptosis. The defective DNA repair by itself is not deadly enough to cause cell death. We could strategically target the damaged site on DNA to achieve synthetic lethality in cancer cells.

![Figure 4 A schematic presentation of synthetic lethality. One mutated gene would not harm the cell, but when both genes were mutated and presented in the same cell, apoptosis would be induced.](image)

The concept of synthetic lethality was first proposed in the 1920s by Dr. Calvin Bridges (19). While crossing fruit flies (*Drosophila melanogaster*), he observed that some combinations of the gene were deadly (19). The concept of synthetic lethality was evolved from Dr. Bridges’ idea: to introduce defects and combine the innate damage in order to paralyze the defect system. Since cancer cells contains large amount of errors, the idea of synthetic lethality was utilized to indirectly take out cancer aberrations that were difficult to target directly (20).

Compared to classical cancer treatments, synthetic lethality has several advantages. Synthetic lethality could be achieved in several different pathways. As stated earlier, cancer cells have different biology compare to normal cells. Focusing cancer cell’s unique biology allows us to target cancer in a more specific manner (2).
For example, adding an outside factor could worsen the mutations in cancer cells to make lethal damage (21). Anti-tumor drugs, such as Bortezomib, Vorinostat and Geldanamycin (see figure 5), have been developed for the cellular targets that are not directly related to tumorgensis. These drugs exacerbate the hallmarks of cancer cells by targeting the already weakened systems, such as the defective repair mechanism and mutated DNAs, in order to induce apoptosis. The drug-gene interactions obtained from studies of these drugs also provided possible explanations of selective sensitivities of cancer cells to different chemotherapeutical agents via synthetic lethality (22).

Figure 5 structures of the three anti-cancer drugs. (A) Bortezomid; (B) Vorinostat; (C) Geldanamycin

A recent development of treating breast and ovarian cancer utilized the idea of synthetic lethality, and significantly prolonged the life-span of the patients. The strategy is to target the mutated BRCA gene and PARP, both of which targets are heavily involved in breast and ovarian cancer (14). The goal is to cripple both repair processes to arrest cell replication in cancerous cells. The HR pathway is not functioning properly in BRCA-mutation associated breast and ovarian cancers. The mutation in BRCA gene makes the cell especially vulnerable to PARP inhibition. Blocking PARP prohibits BER activity, and accumulates DSB. The consequences lead to chromosomal instability, stop the cell cycle, and subsequently cause cell death
(illustrated in figure 6 (23)). Synthetic lethality is a potent method in this case by strategically knocking out both factors to control cell survival. This new strategy was also proved to be a relatively mild and efficient process for cancer treatment (24).

![Figure 6](image)

*Figure 6. A schematic representation of using synthetic lethality to induce cell death. Inhibiting PARP to induce double-strand break and to achieve cell apoptosis. Inhibiting PARP prevent the repair of single-strand break (SSB), and could lead to double-strand break (DSB). The mutation in BRCA disabled the repair of DSB, and leads to cell death.*

Extensive studies of genetic interactions in yeast (*Saccharomyces cerevisiae*) further indicated that synthetic lethality is highly achievable. A recent study showed that in yeast, there were around 5000 different possible synthetic lethal interactions exist within the 74 genes that are involved in genome maintenance (25). Since yeast cellular processes, especially DNA repair systems, highly resemble those of humans, information gained from studying the yeast genome could be beneficial to studies of human (26).

Synthetic lethality can also be utilize to elucidate drug-gene interactions. Yeast is extensively used as a model system to study drug-gene interactions (27). Some of the drugs used in clinic trials show synthetic lethal interactions with the
yeast gene. It is important to identify the specific protein targets of drugs, in order to control the undesirable side effects (28).

Compared to classical cancer treatments, synthetic lethality as a therapeutic strategy against cancer provides more specific targets with higher accuracy. It has the potential to develop a gentler cancer treatment with limited side-effects.

1.4 Target choice for synthetic lethality

There have been many attempts to target the proteins involved in the repair mechanisms, but this strategy will take a tremendous amount of effort with less ideal and slow rewards due to the number of proteins involved in this cellular process (29). Since cancer is associated with multistep alterations of the genome, it is difficult to understand the contribution of individual factors to cancer (30). Also, previous studies have shown that there are only a few key signaling pathways associate closely with cancer cells, and targeting these signal pathways can efficiently control cancer (31). Blocking the damage signal before the cell engages in the problematic repair pathways would be a more promising strategy. This could be achieved by specifically targeting the damage site on DNA.

1.5 Objective of the Project

The goal of this project is to find drug like small molecules (see table 1) that could selectively hide the SSB, so the DNA damage response factors, such as PARP, will not detect the damage and start the DNA repair mechanism. A method that can monitor the binding of drug like molecules on damaged site on DNA is also used. The
improved hydroxyl radical footprinting could accomplish this task. Current methods such as X-ray crystallography and Electrophoretic mobility shift assay have great limitations such as expensive instrumentation, need to grow crystals and unable to detect specificity of the binding process (32). The improved hydroxyl radical footprinting method can monitor the binding process at single-nucleotide resolution, with relatively easy and inexpensive experimental set up (33).

This experiment uses synthetic 29-nt double strand DNA with a single-strand breach between the phosphate backbone of G and A, as shown below, with location of breakage highlighted in red:

3’-CCGTGGTCCAGCCGCTCGGAGTAGACTGTTTTTTTTTTTTTTTTTT/5AmMC6T–5’  (A)
5’-GGCACCAGGTCCGCGAGCCTCAGCCTGAC–3’  (B)

Figure 7 this illustration shows a close up view of the breakage between guanine and adenine. There is a breach in the phosphate backbone.

1.6 Table of tested ligands
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<th>Ligand</th>
<th>Structure</th>
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<tr>
<td>EtBr</td>
<td><img src="image" alt="EtBr Structure" /></td>
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</table>
1.5 Footprinting DNA-ligand Complex Using Improved Hydroxyl Radical Reaction

Hydroxyl radical footprinting is an excellent method for studying the binding site of DNA-ligand complexes. The highly reactive hydroxyl radicals can cut the DNA at a non-sequence specific manner, and also yield to single-nucleotide resolution. Hydroxyl radicals cleave DNA sequences by attacking the 5’-hydrogen atom of deoxyribose to cause formation of a single nucleotide. It can also attack the 4’-hydrogen on deoxyribose sugar and yields to two cleavage product: 3’phosphate and 3’-phosphoglycolate. The formation of the undesired products can interfere with the ligand binding monitoring, and they are usually displayed as splitting of bands on the gel (32).

The DNA sequences that are bound by ligand will be protected from hydroxyl radical attach. The bound sequences will be visualized on an acrylamide denaturing gel, and the protected region will have less cleavage in the lane (32).
An improved hydroxyl radical reaction modified the DNA of interest and improved the protocol to enhance product yield and resolution of footprinting. The testing DNA sequence is labeled with dTₙ tails with a modified amine 5AmMC6T, which allows the covalent bonding of Oregon Green fluorescence labeling (33). The dTₙ tails also allow the hydroxyl radical product to co-precipitate with poly(A), and result in a better yield of cleavage products by increasing the length of the fragments. The use of a fluorescent dye is a convenient and stable technique. It avoids the traditional radioactive label P³² and the limitation of its short half-life. The improvement for this protocol is summarized in figure 10 (32).
Hydroxyl radical reaction only requires commonly used lab equipment and relatively inexpensive reagents. Fenton reaction, a simple chemical reaction, is used to generate hydroxyl radicals \( \cdot \text{OH} \). As shown in the following scheme:

\[
[\text{Fe(EDTA)}]^2^- + \text{H}_2\text{O}_2 \rightarrow [\text{Fe(EDTA)}]^- + \text{OH}^- + \cdot \text{OH}
\]

After the free radical reaction, the products are analyzed by denatured gel electrophoresis. A fluorescent sensitive scanner is then used to visualize the footprint in the resulting gel.
II. **Methodology and Materials**

2.1 **DNA samples**

The DNA nucleotides were obtained from Integrated DNA Technologies (IDT) in HPLC purified form. Sequence A was ordered with dTₙ tail at the 5’ end. The complementary strand, sequence B was made of two separate orders, sequence C₂ and sequence C₃. Phenol-chloroform extractions were performed on the DNA sample to extract the undesired free amine from the dTₙ tail. The free amine from DNA sample (250 µM) was extracted by 100 µL of phenol, then twice with chloroform. Ethanol precipitation was then performed by adding 200 µL of chilled ethanol. After the DNA sample was dried by a speed vac, it was dissolved in 100 µL of phosphate buffer, 2.5 mM Na₂HPO₄, pH 7. Then the sample was precipitated in 20 µL of 3M NaCl solution and 500 µL of chilled ethanol. The samples were centrifuged at 13,000 rpm for half hour, and the supernatant was removed. The precipitation process was repeated three times. After the final sample was dried using speed vac, 100 µL of double-distilled, autoclaved water was added to dissolve the sample. The absorption of the sample at 260 nm were determined by the Nanodrop spectrometer (Thermo Scientific, 2000c). The concentration of the DNA was calculated using Beer’s Law: \( A = \varepsilon l C \), where \( A \) is absorbance, \( l \) is path length (0.1 cm for Nanodrop spectrometer), \( \varepsilon \) is extinction coefficient, which is assumed to be 365,900 L/(mole·cm) (35) and \( C \) is concentration.

2.2 **Oregon Green (fluorescence) labeling**
The fluorescence labeling was performed on chain A. The dried DNA pellet of chain A was dissolved in 100 µL of doubly distilled, autoclaved water. Then, a solution of 250 µg of Oregon Green 514 carboxylic acid succinimidyl ester (Invitrogen) in 14 µL DMSO and 75 µL of 0.1 sodium tetraborate at pH 8.5 was made, and 14 µL of Oregon Green 514 solution was added to the DNA sample. The sample was incubated overnight at 310 °K on a shaker with low oscillation. Three ethanol precipitations were then performed to remove the free dye, and the sample was re-dissolved in phosphate buffer. The nanodrop spectrometer was employed to measure the percent labeling, by comparing the absorption at 260 nm and 510 nm, which are the wavelength for DNA ($\varepsilon = 365,900 \text{ L/(mole}\cdot\text{cm})$ (35)) and Oregon Green 514 ($\varepsilon = 70,000 \text{ L/(mole}\cdot\text{cm})$ (36)), respectively. The extent of Oregon Green labeling was found to be 75%.

2.3 DNA sample preparation for binding experiment

The DNA duplex was prepared by adding the solution of chain A (fluorescence labeled), chain C$_2$ and chain C$_3$ in 100 mM KCl, 2.5 mM phosphate buffer to a final concentration of 2.5 µM each. The mixture was heated at 363 °K for 7 minutes, and gradually cooled to room temperature. The bulk duplex DNA solution was separated into small tubes to achieve a final duplex DNA concentration of 2.5 µM. DNA was then incubated with ligands (see table 1) at 2 µM, 5 µM, 10 µM, 20 µM or 40 µM final concentration.

2.4 Hydroxyl radical cleavage reaction
The hydroxyl radical cleavage reaction was performed by adding each DNA sample to 2 µL of 10 mM Fe(II)/20 mM EDTA solution, 100 mM sodium ascorbate and 0.04% H₂O₂. The reaction was allowed to proceed for 2 minutes, and then quenched with 10 µL of 230 mM thiourea. The quenched sample was added with 10 µL of 30 mM poly(A), followed by 4 hours of ethanol precipitation on ice. The samples were centrifuged at 13,000 for 30 minutes and the ethanol supernatant were removed, allowing the precipitated DNA on bench to dry overnight.

**2.5 Dimethyl sulfate (DMS) footprinting**

To 2.5 µM of Oregon Green labeled single strand DNA, 2 µL of 0.1 M dimethyl sulfate (DMS) was added and incubated at room temperature for 10 minutes. Then 10 µL of 30 mM poly(A) was added and the DNA was precipitated with ethanol and dried as discussed in section 2.4.

**2.6 Pyrrolidine digestion**

The products of the hydroxyl radical cleavage reaction were dissolved in 40 µL of 0.1 M pyrrolidine solution and heated at 363 °K for 30 minutes, cooled and centrifuged for 1 minute. The samples were dried and lyophilized using a speed-vac.

**2.7 Gel electrophoresis imaging**

The pyrrolidine digested products were dissolved in 6 µL of double-distilled, autoclaved water and 6 µL of 10 M urea, and heated for 4 minutes at 363 °K and cooled for 1 minute. The gel used was 20% polyacrylamide denaturing gel, with
dimension of 20 cm x 40 cm x 1 mm. The gel was pre-run in TBE buffer (0.1 M Tris base, 0.1 M boric acid, and 1 mM EDTA, pH 8) for 40 minutes to 1 hour before loading the sample DNA. The samples were loaded on the gel and electrophoresis were performed at 50 W for 8 hours, or 60 W for 5.5 hours.

The gel was imaged using a GE Typhoon Trio scanner (Amersham Biosciences). The gels was scanned at the green fluorescent-excited mode at 532 nm with an emission filter at 526 nm and the photomultiplier at 600V.

2.8 Quantification

The gel image (figure 11A) was analyzed by Semi-Automated Footprinting Analysis software (SAFA v1.1) (37). The value of band intensities obtained by SAFA was exported on excel (seen in figure 11B). Each band is identified base on DMS footprinting results. DMS specifically cleaves guanine, and it is used as an indicator for nucleotide identification on the gel (38). The intensity of each lane was summed and then normalized. The percent change of cleavage was calculated and presented as a histogram in figure 11C. The algorithms for the calculation are showed in figure 11C, step 3. The results from the ligand experiments are shown in the Results section. Each experiment was repeated at least 4 times to eliminate human errors and to ensure reproducibility.
Figure 10 A: Gel image of tetralysine binding the DNA. B: Numerical presentation of data from A, analyzed by SAFA. C: Algorithm for each data quantification for each step. Step 1 is to transfer data into numerical value. Step 2 is to sum the intensities from each lane and normalized then. Step 3 is to calculate the percent change of cleavage of the ligand. D: A presentation of final result using histograms.
III. Results and Discussion

3.1 Attachment of dT tail and poly(A) co-precipitation

There are two major challenging issues associated with hydroxyl radical cleavage and ethanol precipitation. Hydroxyl radicals as cleavage agents could destroy the fluorescent dye as well as produce over cleavage of DNA, which would disrupt band intensities (32). Ethanol precipitation could result in loss of cleavage products as it extracts contaminants such as quenchers and reactants. Previous research in our lab showed that by attaching dT tails to the sequence of interest and adding relatively high concentration of poly(A) after the cleavage reaction could eliminate these problems (33).

The attachment of dT tails can increase the length of the smallest nucleotide, which enhances single nucleotide resolution and prevents too much cleavage by hydroxyl radicals (39). Also, pyrrolidine digestion can restore the fluorescent level (33). The efficiency of ethanol precipitation depends on the length of sequences, and it is also well known that adding polynucleotide could increase the yield (32). The addition of the polynucleotide poly(A) before the ethanol precipitation step allows co-precipitation with the cleaved product with dT tails to increase yield. Previous research in our lab also determined that 3 mM of poly(A) was enough to achieve a better yield and gel resolution (33).
3.2 Cleavage of SSB sites in the presence of polyamine and polycations

The effects of highly positive charged molecules, spermidine, spermine and tetralysine binding to DNA with SSB were examined.

Spermidine and spermine are polyamines present in various organisms, and are essential growth factors in some bacteria. They are polycations at neutral pH and thought to stabilize the helical form of nucleic acid (40). Tetralysine is similar to the polyamines, as it has multiple cationic sites and binds to negatively charged DNA (41). The high positive charges present in these molecules make them the possible binding candidates of the highly negatively charged SSB site.

The footprint result shown in figure 12 indicated that spermidine binds strongly at the SSB (the SSB site is highlighted in yellow) in the order of 60% of cleavage change, when the ligand concentration is at 5 µM and 20 µM. However, the histogram also indicated that spermidine affects strongly at several other sites at the order of more than 40%. The experiment was repeated more than 10 times, and the results indicated that spermidine did not selectively influence the SSB site. It shows unspecific binding to DNA. The footprint results of spermine binding DNA is less prominent, shown in figure 13. It appears that the cleavage is at the order of 15%, which is an insignificant percentage change and could just be the noise. The footprint result of tetralysine is shown in figure 14. Previous study has suggested that lysine conjugates were efficient at recognizing DNA damage (42). Significant cleavage changes have observed from tetralysine binding the SSB site, but also at other sites.
The result is then compared with tetralysine binding fully complementary DNA, and the showed a similar protection pattern, which also indicated unspecific binding of DNA. Cleavage data generated from this experiment did not show that tetralysine can specifically recognize DNA damage.

Figure 11. The histogram illustrates the percent cleavage change at the SSB site (yellow) in the presence of spermidine at 5 µM, 10 µM and 20 µM concentration.

Figure 12. The histogram illustrates the percent cleavage change at the SSB site (yellow) in the presence of spermine at 10 µM and 20 µM concentration.
Figure 13. The histogram illustrates the percent cleavage change at the SSB site (upper histogram, yellow) and fully complemented duplex DNA (no SSB, lower histogram) in the presence of tetralsine at 2 µM, 10 µM and 20 µM concentration.

3.3 Cleavage of the SSB site in the presence of minor groove binders

DNA minor groove binders, DAPI, Hoechst33258 and netropsin were tested next. The footprint results are indicated in figure 15, 16 and 17. A previous study in our lab suggested DAPI and Hoechst33258 showed significant effects on CC mismatch (33), but binding experiments with SSB DNA showed insignificant cleavage changes and less specific recognition at the site of interest. The change at the SSB site was less than 20%, and more significant changes (over 25%) were observed at various other sites both upstream and downstream of the SSB.
Hoechst33258 showed a stronger cleavage change (figure 16) upstream of the SSB. A fully complementary DNA was tested with the ligand in comparison with the SSB DNA, but the results didn’t show specific binding. Although there were protection pattern differences at various sites, the site of interest was not affected.

Footprint results in figure 17 shows the footprint results of netropsin, an antiviral agent (43). Ward et al. indicated netropsin discriminated 5’-TA-3’ regions of DNA due to DNA structural alteration from Adenine-adenine clash (43). Our DNA does not contain 5’-TA-3’ sequence, so netropsin was not expected to show specific binding at the site of interest. A small overall cleavage change of less than 20% is shown in figure 17, which is not significant enough for ligand binding site evaluation.

CCGTGGTCCAGCGCTCGGAGTGAGACTGGCACCAGGTCGGCGAGCCTCACTCTGAC

Figure 14. The histogram illustrates the percent cleavage change at the SSB site (yellow) in the presence of Dapi at 5 µM, 10 µM and 20 µM concentration.
Figure 15. The histogram illustrates the percent cleavage change at the SSB site (upper histogram, yellow) and fully complemented duplex DNA (no SSB, lower histogram) in the presence of Hoechst33258 at 5 µM, 10 µM and 20 µM concentration.
3.4 Cleavage of a SSB site in the presence of intercalators

The effects of the DNA intercalators ethidium bromide (EtBr) and berenil on SSB DNA were investigated. Figure 18 shows that EtBr affects the majority of the sequence with significant change at the order of 25%. However, the change was less prominent at the SSB site. It appears that EtBr does not specifically mask the SSB, but discriminates against it by showing weaker affinity compared to the rest of the sequence. This results is expected, since EtBr intercalate between DNA base-pairs by hydrophobic interaction and stacking interaction. The opening created at the SSB site alters the geometry of the base-pairs presented. As a result, EtBr was not expected to insert itself in between the base pairs. Figure 19 shows berenil binding a SSB. The percent change in cleavage is less than 10% at the SSB site, which indicates the binding might not happen at the site of interest.
Figure 18. The histogram illustrates the percent cleavage change at the SSB site (yellow) in the presence of berenil at 10 µM, 20 µM and 40 µM concentration.
IV. Conclusions and Future Directions

In this research we have presented the effects of polyamines, polycations, minor groove binders and an intercalator on the SSB by using the improved hydroxyl radical cleavage reaction and DNA footprinting. The modifications were made on the sequence of interest by addition of dTₙ tails at the 5’-end and co-precipitation of poly(A) to enhance the yield of cleavage products and fluorescent signals. The footprinting results indicated that other than ethidium bromide, most of the ligands tested did not show specific effect toward SSB. More ligands will be investigated.
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References:


