ABSTRACT

Polyamide-Chlorambucil Conjugates

Polyamides are chemically engineered molecules made up of sequences of pyrolle and imidazole rings. in particular sequences, these molecules have been shown to have a very high affinity and specificity for nucleic acids, and can therefore be arranged to bind a specific sequence of DNA. By combining such a compound with a known anti-cancer agent, Chlorambucil and using microarray technology to query the specificity and affinity with which this conjugate binds DNA, and in particular which sequences are bound.

Appesh Mohandas / Biochemistry
Author Name/Major

Author Signature

6/16/08
Date

Mentor Name/Department

Mentor Signature CLW

COVER SHEET

TITLE: Polyamide-Chlorambucil Conjugates

AUTHOR'S NAME: Appesh Mohandas

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Dr. Aseem Ansari

DEPARTMENT: Biochemistry

MENTOR(2): Dr.Christopher Warren

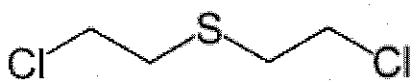
DEPARTMENT(2): N/A

YEAR: Spring 2008

Background

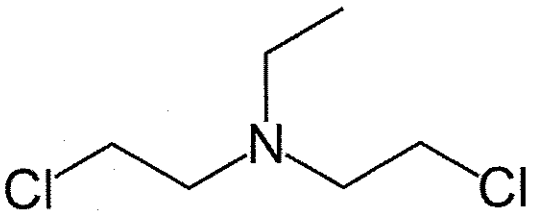
Nitrogen mustards and the development of Chlorambucil.

The origins of Nitrogen mustards lie in Sulfur mustards, with a structure noted in figure one below. Sulfur mustards came into prominence in the First World War, about thirty years after their original development. It was observed that these sulfur mustards had the potential to inhibit cell division, but also that they where very toxic even at relatively low concentrations.



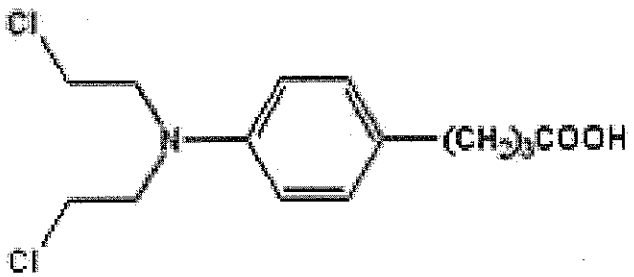
Chemists developed a related class of mustards in which the Sulfur atom was replaced with Nitrogen (figure 2 below), reducing the electronegativity of the central atom and thus reducing the molecule's tendency to form the electrophillic aziridinium that accounts for its high reactivity. This modification also reduced the toxicity of the mustard, and made it suitable for clinical uses. Modified nitrogen mustards are used as therapeutic agents even today.

Chlorambucil (figure 3 below) is such a modified agent that first came in to clinical use in 1948[], and is used today to treat diseases like Hodgkins Lymphoma[]. Their chemical



uniqueness has been explored in some detail, and general patterns have been identified. In general, mustards work by hydrolysis of the terminal Chlorine to a chloride ion in water, and the immediate formation of a cyclic aziridinium ion with the carbocation attacking the central Nitrogen atom to quench the positive charge. This creates a highly electrophillic site which is open to nucleophillic attach for example by atoms on the phosphate backbone of DNA.

The modification of Sulfur mustards to Nitrogen mustards decreased the electrophillicity of the mustard slightly, but nucleophillic attack was still possible by a variety of molecules in the cell. Assuming that there is a wide range in strength of the nucleophiles in a cell, having a compound that is highly electrophillic means that there are many possible attacking groups. This meant that the Nitrogen mustards could not be used as a specific pharmacological agent without the risk of a wide range of side effects.The development of a slightly less electrophillic molecule was necessary because this would decrease the number of possible attackers and thus decrease the number of possible side reactions or side effects. The solution was found by the addition of an aromatic ring, forming 4-[bis(2-chlorethyl)amino]benzenebutanoic acid, or Chlorambucil



(figure 4). This ring gives the compound a greater capacity to stabilize the carbocations that result from the dissociation of the Chloride ions. Also, the ring has enough of an electron-donating capacity to inhibit the cyclization of the ethylene cation.[] Thus, Chlorambucil differs from the most general form of Nitrogen mustards in that the carbocations are free-floating rather then in highly sterically stressed three member ring structure. This means that the Chlorambucil molecules are less electrophillic than their parent Nitrogen mustards. While it still may alkylate a wide range of molecules, are suitable for therapeutic purpose, and Chlorambucil has been in clinical use since 1948.

It was shown clinically that Chlorambucil was a promising anti-tumor drug, and that its alkylation target was DNA. Furthermore, work has been done that shows which sites in DNA, and which sorts of region Chlorambucil has a higher affinity for. Since the molecule needs a nucleophile to attach to the carbocation and form the alkylation product, it is no surprise the

more nucleophillic base pairs form substrates with Chlorambucil. It has been shown that Chlorambucil alone has an affinity for the N7 residue of Guanine, which it attacks from the major groove. It has also been shown that the molecule will bind the N3 of Adenine. Furthermore, Chlorambucil likes to bind Guanines that lie within stretches of Guanine residues. The explanation offered by Kohn, Hartley, and Mattes says that the strongly negative molecular electrostatic potential of these sequences may account for this since the reactive group's positive charge may be drawn to these electronegative regions.

Crosslinking is possible with Chlorambucil as well, but there is minimal published data on this. Kohn et al reported in 1987 that both inter- and intra-strand were possible. Intrastrand crosslinks could occur at regions with sequential G's, and interstrand crosslinks might be possible at 5'-GC-3' regions, but Kohn reported only weak reactivity here.

Polyamides

Long in development by Peter Dervan at Caltech, polyamides have evolved into a potential means of regulating gene expression. Variations in the sequence of Imidazole (Im) and Pyrolle (Py) rings that are adjacent across double stranded DNA give the hairpin structure a well defined specificity to a DNA sequence, and these generally have affinities in the subnanomolar range, similar to the affinities of DNA-binding proteins. However, whereas proteins bind in the major groove, polyamides have been shown to bind in the minor groove

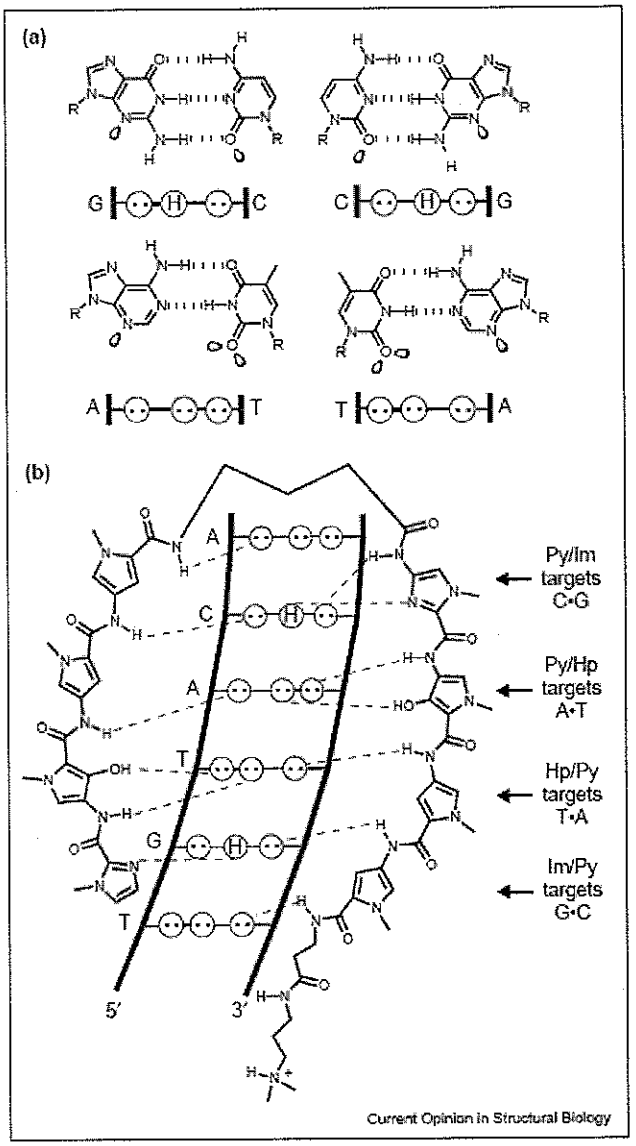
1. Synthesis

Synthesis of the polyamides first used a liquid-phase method, which required months for the completion of one set of polyamides. The solid phase methods which were developed later and

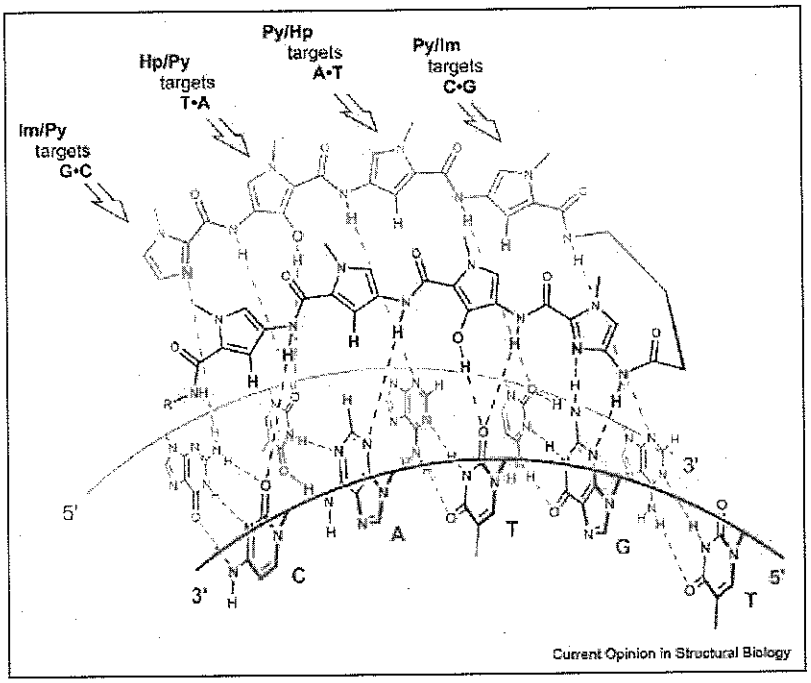
are used today take only days to complete and have allowed researchers to test their hypotheses for the specificity and affinity of different arrangements of the amide-linked structures. Some of the most common solid phase methods that are in use today are the Boc-β-Ala-PAM,which uses Boc-protected monomores,and Fmoc-β-Ala-PAM-Wang. One of the drawbacks to these methods is that they both leave a T/A ('W') selective β-Alanine residue at the C-terminus tail. This clearly limits the specificity that one can achieve with the polyamides. A solution to this problem was shortening the tail, and while minimal shortening was achievable via post-synthesis chemistry, the adaptation of another resin known as the Kaiser oxime resin was adapted to polyamide synthesis. This has allowed for the shortening of the C-terminal tail and the synthesis of polyamide that can bind G/C base pairs while maintaining high affinities for their consensus sequence.

2. Sequence recognition

The binding of polyamides is dictated by non-covalent interactions between the polyamide and the specific DNA bases. Furthermore, these non-covalent interactions are dependant upon the arrangement of structures that are opposite to one another across the hairpin configuration. For example, the Im/Py arrangement targets G/C and that Py/Im arrangement targets C/G. this specificity is conferred by three important stabilizing hydrogen bonds. The N3 of the imidazole ring shares an H with the N of the 2-amino groups on guanine, and the N-terminal amide nitrogen shares its H with the N6-carboxyl group of guanine. The opposing Pyrolle ring's N-terminal amide Nitrogen shares its Hydrogen with the Oxygen in the carboxyl group at the N2 position.



Molecular recognition of the minor groove of DNA. **(a)** Minor groove hydrogen-bonding patterns of Watson–Crick base pairs. Circles with dots represent lone pairs of N(3) of purines and O(2) of pyrimidines, and circles containing an H represent the 2-amino group of guanine. The R group represents the sugar–phosphate backbone of DNA. Electron lone pairs projecting into the minor groove are represented as shaded orbitals. **(b)** Binding model for the complex formed between ImHpPyPy-γ-ImHpPyPy-β-Dp and a 5′-TGTACA-3′ sequence. Putative hydrogen bonds are shown as dashed lines.



Schematic of a Py-Im polyamide–DNA complex. Perspective view into the minor groove with the curvature lessened and the twist between the base pairs removed, illustrating hydrogen-bonding patterns and shape complementarity. The R group represents β-Dp. This figure presents an alternative view of the complex shown in Figure 1b. The phosphate backbone of each DNA strand is indicated by a solid curved line with the 5' and 3' ends noted.

It was discovered that there is a limit as to how many Im or Py rings could be connected before the molecule could no longer bind DNA due to an inability to model its curvature. To solve this problem, Dervan's group inserted a β-Alanine into the ring to relieve this steric problem. Binding rules have also been developed for β-Alanine/Imidazole and β-Alanine/Pyrolle rings The a β-Alanine/Imidazole (β/Im) complex

Table 2. Pairing code for minor groove recognition[a]

Pair	G•C	C•G	T•A	A•T
Im/Py	+	–	–	–
Py/Im	–	+	–	–
Hp/Py	–	–	+	–
Py/Hp	–	–	–	+

[a]Favored (+), disfavored (–).

Table 3. Pairing code β-alanine (β), Py/Py and Im/Im pairs.[a]

Pair	G•C	C•G	T•A	A•T
Im/β	+	–	–	–
β/Im	–	+	–	–
Py/β	–	–	+	+
β/Py	–	–	+	+
β/β	–	–	+	+
Py/Py	–	–	+	+
Im/Im	–	–	–	–

[a]Favored (+), disfavored (–).

binds C/G, β/Py binds T/A or A/T, and β/β binds T/A or A/T as well.

The technology of polyamide synthesis and the understanding how they can be manipulated to bind a specific sequence of DNA has progressed to the point where polyamides can be engineered to bind an 11-16 base pair region of DNA. According to a 2005 Nature Reviews Cancer paper by Neidle and Thurston, this is approximately the length that such molecules will need to target in order to be effective individual genes in DNA.

Polyamide-Chlorambucil Conjugates

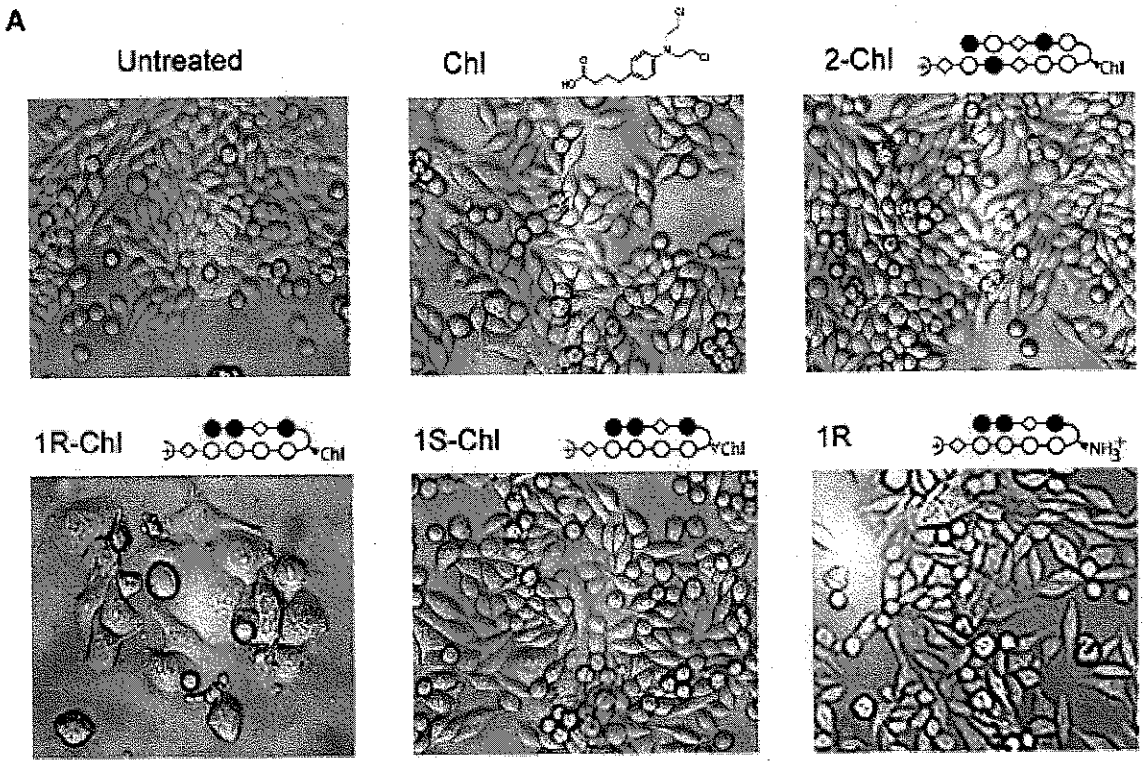
The most promising proposed use of the polyamide technology is in gene regulation, and much progress has been made in this area already. These small molecules have the distinct advantage of being able to localize in the nucleus. One of the technologies that has been developed is to conjugate polyamides with DNA alkylators such as those that are used in cancer therapeutics.

Joel Gottesfeld's group at the Scripp's Institute has synthesized such a compound that conjugated a polyamide with chlorambucil, a common anti-cancer agent that alkylates DNA (described above). For their experiments, the group has conjugated chlorambucil with multiple different polyamide structures, one being a biologically interesting. This compound is known as 1R-Chlorambucil. The sequence of the structure is Im-Im-β-Im-α-Py-Py-Py-Py-β-Dp (Im= Imidazole, β=β-Alanine, α= and α turn, and Py=Pyrolle) and the Chlorambucil moiety is attached at the α turn with R stereochemistry. According to the literature, this structure is supposed to be specific for the sequence 5'-WGGWGW-3', which is consistent with the base pairing rules that are described above. This sequence has been shown to exist in the coding and promoter regions

of the H4c histone protein. This gene is shown to be highly expressed in some types of cancers, especially colon cancer. In colon carcinoma cells, the sequence 5'-WGGWGW-3' appears 4 times in the coding region of the H4c gene, and once is the promoter region. According to DNase Footprinting by Gottesfeld's group, 1R-Chl binds well to one of the sites in the coding region, but only weakly to the site in the promoter region. It has been shown that out of all the polyamides tested, only 1R-Chl is able to alkylate the DNA of the histone protein, and halt the cell in G2/M phase. While some of the other polyamides are cytotoxic or entirely ineffective, 1R-Chl has been shown to be cytostatic, inhibiting the action of RNA polymerase.

Most experiments with 1R-Chl have been done on the SW620 colon carcinoma cell line and they have been able to visualize the effect of this specific alkylator at the cellular level. Cells exposed to 1R-Chl appear to swell, and it is clear that the chromatin is not as condensed as cells treated with control molecules. {FIGURE} These experiments show that 1R-Chl does have a cytostatic effect on the cells that the other molecules do not, and the swelling of the cells and the lack of condensation of the nuclear chromatin certainly indicate that damage to a histone protein

may be involved.

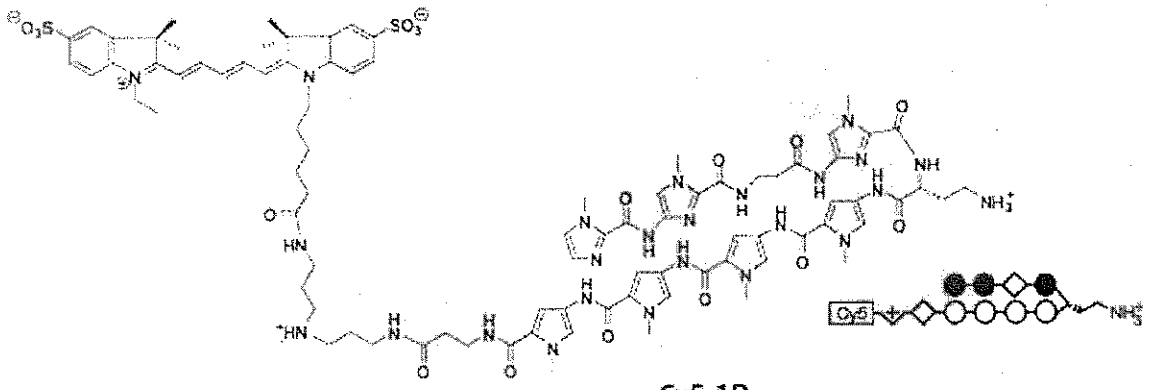


However when two slightly different polyamide conjugates were exposed to mice, a very drastic event was observed. Mice that were exposed to the polyamide 1R-Chl with and α-diaminobutyric acid (α-DABA) conjugated chlorambucil did not differ that much from control mice, but a polyamide with the same sequence of ring structures in the hairpin and with a γ-DABA turn instead of the α-DABA turn proves to be lethal in mice. A chemical explanation was on offered in the paper, but one may suspect that the gamma turn allows for a more widespread alkylation than the alpha turn, or even that the different type of conjugation confers a specificity that localizes the PA-Chl conjugate to a sequence of great importance to the viability of an organism. However, it has been shown that the conjugate with the gamma turn is much more a much more active and much less specific agent than that with the alpha turn.

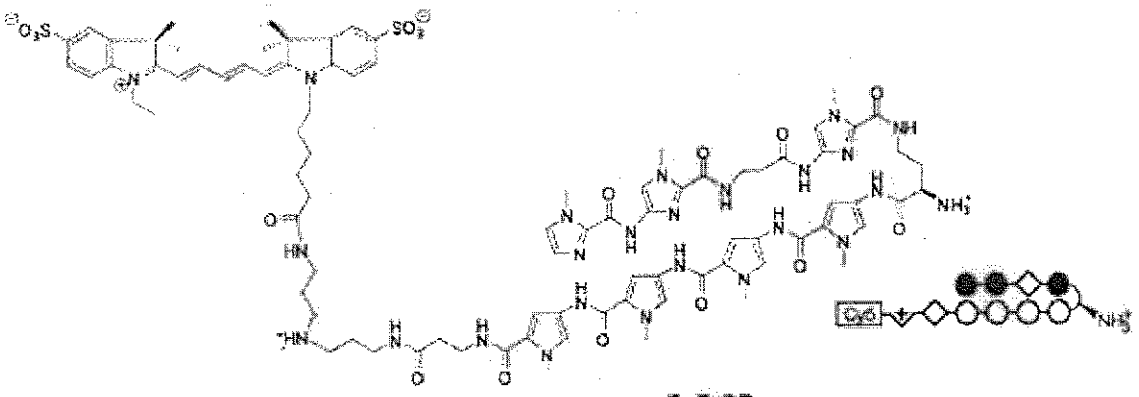
Introduction

Polyamide-chlorambucil compounds have shown significant promise as a specific DNA alkylator that with take the field of cancer therapeutic on large step towards localizing DNA-alkylating therapeutics to specific genes and thus decreasing the side effects of current therapeutic solutions. However, one must be cautious about the degree of specificity that a polyamide-chlorambucil molecule is given. If there are three million base in the human genome, and the polyamide is specific to the sequence 5'-WGGWGW-3', given that W=A or T, there are about 6.5 million locations where this sequence will appear. Given this ubiquity, it is important to understand where these sequences may lie, and what genes other than the H4c gene of the H4 histone protein might be affected by exposure to 1R-Chl. In order to query this, the entire genome needs to be surveyed and binding data must be compared to published genomic sequencing information. This project was a clear candidate for the Cognate Site Identifier microarray for multiple reasons. First, previous experiments lend a great deal of credibility to the CSI array's ability to elucidate the affinity and specificity with which DNA-binding molecules bind DNA, especially with polyamides and normal B-form DNA. Second, the CSI array can represent every 10mer DNA sequence that exists in any genome, giving researchers the power to analyze this polyamide-chlorambucil conjugate against the organism of their choice. Lastly, the CSI array is able to comfortably compensate the consensus sequence as well as the alkylation site of the PA-Chl conjugate. In order to understand the binding of the polyamide-chlorambucil compounds, my project has focused on comparing the the DNA-binding affinity and specificity of four different polyamides, 1R, 8R, 1R-Chlorambucil, and 8R-Chlorambucil. The differences between the two compounds are that 1R has an α-DABA turn, whereas 8R has a γ-DABA turn. In this project, I will compare the specificity and affinity of all of these compounds in order to

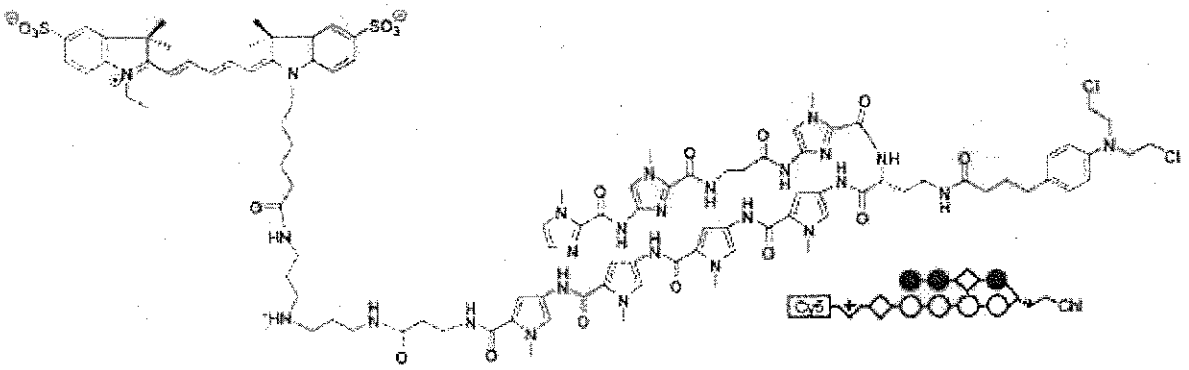
determine the differences between each of them, as well as how well each of these are able to bind to their consensus sequecnce. This information will be gathered using the CSI microarray technology, and it will be analyzed as to how well the data correlates with the structure of the polyamide, with published work on the polyamide, and how Chlorambucil affects the binding of the polyamides. Furthermore, we will make comparisons between the mouse genome and the binding properties of the alkylating polyamides to determine what promoter sequences or genes other than the H4c gene and promoter sequence may be affected by the specific binding of these polyamides.



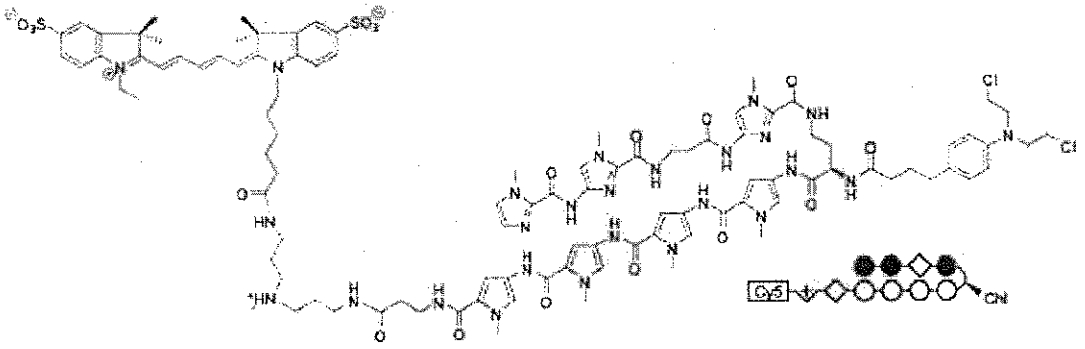
Cy5-1R



Cy5-8R



Cy5-1R-Chl



Cy5-8R-Chl

Methods

Synthesis and solution of polyamides

Polyamides were synthesized by Michelle Farkas in the Dervan lab at Caltech. They were each provided in duplicate aliquots of 50nmol aliquots, and they were brought up to 10 μM aliquots of 50 μL each and stored in the dark due to their Cy5 label.

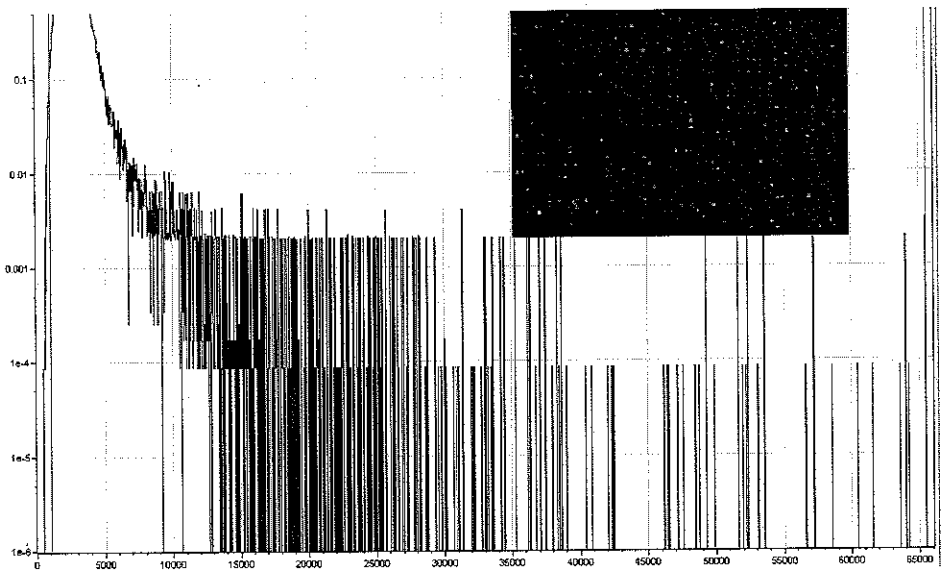
CSI array experiements

1. 1R and 8R

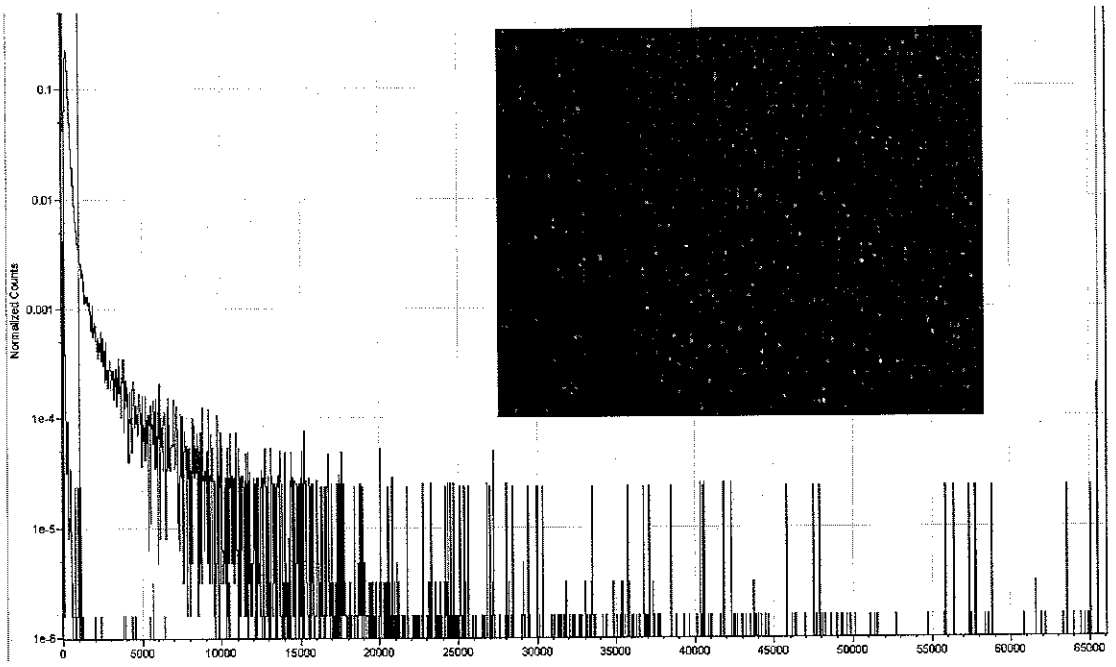
Given that these compounds were much more simple than the chlorambucil conjugates, and that there was no worry of damaging the array as severely as the alkyating polyamides would, experiments began with these compounds. The first step was to find the appropriate buffer. Literature reported that most experiments with the chlorambucil conjugates and their controls had been done in 25mM Tetraethylammonium (TEA), 1mM EDTA at a pH of 7.2. When the 1R and 8R polyamides were diluted in this buffer and tested on a blank slide, the movement of the solution within the array chamber was to poor for array experiments.

The next best solution was to find a buffer that seemed to work with many experiments, and contained fairly common levels of salts. The Hox buffer in the lab was determined to be such a buffer, and it was used to run the 1R and 8R experiements. Also, it was determined that blocking with 2.5 percent milk before hybridization improved the movement on the arrays. Results were successful, as very high binding was observed at 1nM concentration. Due to the lower quality of these particular arrays, it has not yet been possible to extract the data for 1R. While 8R has been extracted, it has thus far been difficult to get a binding motif for this compound. This, however,

is consistent with published data for the compound, in that 8R is known to bind with much less specificity than 1R.



1nM 1R in Hox buffer



1nM 8R in Hox buffer

2. 1R-Chl and 8R-Chl

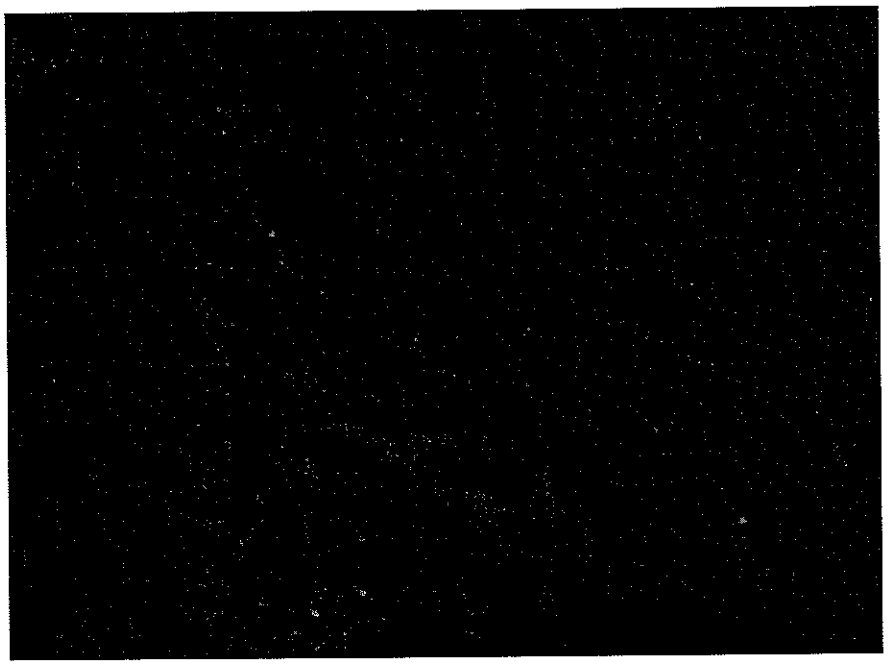
The next step as to get CSI data for the polyamide-chlorambucil compounds. It was suspected that these compounds would be a little more tricky to work with, given their alkylating moiety. For this reason, a lot of effort was put in to understanding the chemistry behind Chlorambucil and related compounds, as well as understanding what conditions previous experiments had used in their work with these conjugates.

The first experiment with 1R-Chl was run in PBS, since it was thought that Hox may quench the alkylating property of chlorambucil. In this experiment, the array was first blocked with 2.5% milk for 1 hour, then 1nM 1R-Chl was hybridized in PBS for one hour and then scanned. While there was some trouble with how well 1R-Chl was moving in PBS on the array, the lack of specific binding is consistent with later experiments that gave no data and showed no specificity as well.

1nM 1R-Chl in 1X PBS

The assay we had decided to use in order to determine whether or no there was alkylation by chlorambucil was to wash the array after hybridizing with the conjugate and scanning it

before and after the strip. The image above represents the first scan, before stripping the array. After stripping the array, there were spots that may appear to bind specifically to some features, these are actually due to spatial effects and appear to be individual pixels rather than actual features. However, most of the large blotches have been stripped away.



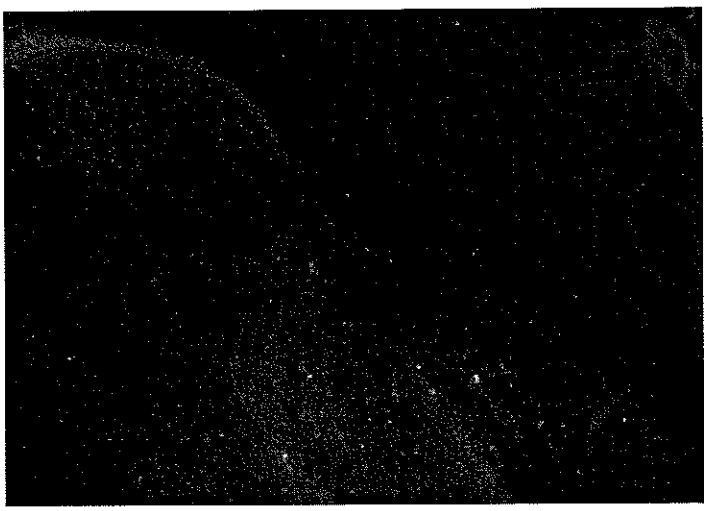
1nM 1R-Chl in 1X PBS, after stripping

Given these difficulties, the next step I took was to compare 1R-Chl with two different buffers, in order to determine which buffer allowed for the hybridization conditions that would allow the conjugate to move best. It was determined that between Hox and PBS, PBS allowed for better movement and would make a better buffer.

It was also a concern whether blocking with milk was resulting in an unfavorable surface condition for the array that was causing a lot of the polyamide to bind the surface instead of the features. Also, while PBS did allow for better movement during hybridization, we had previously seen good results with Hox for the non-conjugated compounds. In order to investigate this, I

used two blank arrays to test the effects of blocking with milk, and I diluted 1R-Chl in 1X Hox to 1nM for hybridization. The array that did get blocked with milk showed a lower signal than the array that did get blocked, indicating that blocking may actually be better at decreasing background binding than not blocking.

The question at this point in the project was still what buffer to use, and I found a paper from Wurtz and Dervan that used 10 mM Tris-HCl, 10mM KCl, 10 mM$MgCl_2$, and 5mM CaC l_2, and a pH of 7.0. In this buffer, I ran 1R-Chl at 1nM, but the results were similar to the previous 1R-Chl experiment. There was a lot of bad spatial effects, and no specific binding visible.



1nM 1R-Chl in Wurtz buffer (0 mM Tris-HCl, 10mM KCl, 10 mM$MgCl_2$, and 5mM CaC l_2, and a pH of 7.0)

Given the success of the non-conjugated compounds in the Hox buffer, I decided to try 8R-Chlorambucil at 1nM. Despite the fact that the solution in the chamber was absolutely resistant to moving during hybridization, and that I ended up having to pipet the solution in and out of the chamber, exposed to light for a large part of the experiment, I saw binding on the

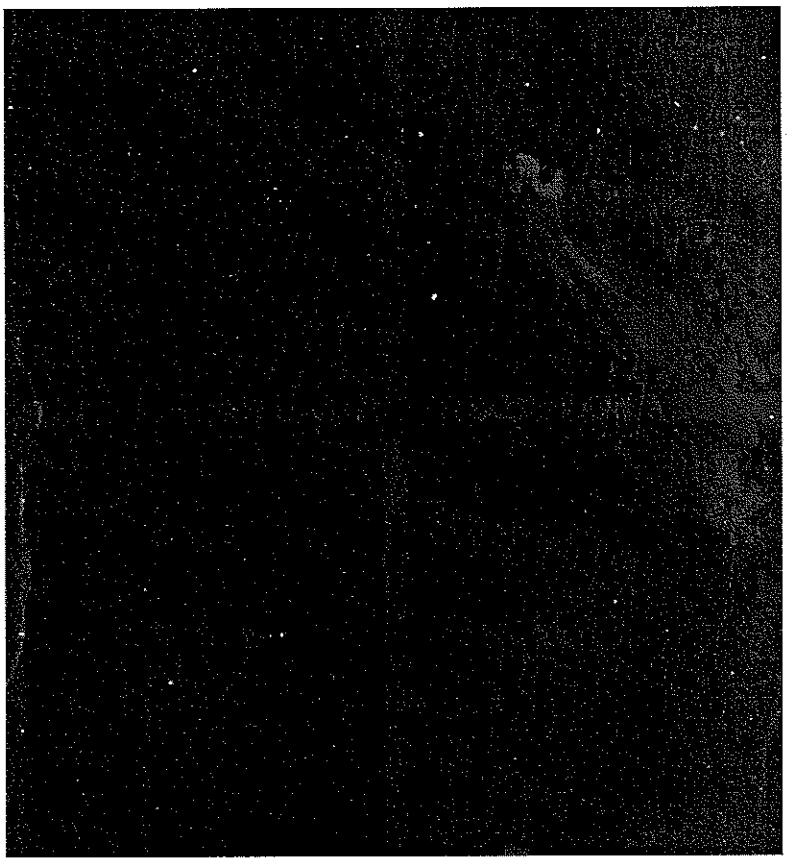
array! About 80% of the array had horrible spatial effects, but there were some spots that clearly showed specific binding. However, with the conditions under which the experiment was run, it was difficult to be confident in these results.

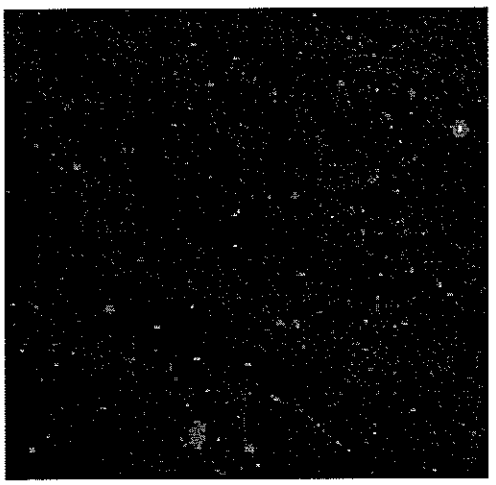
Molecule	Abs @ 310 nm	Conc (uM)
1R	0.0084	13.8
1R-Chl	0.0223	37.1
8R	0.0145	24.1
8R-Chl	0.0132	22.9

Now that I had seen specificity (to some degree) with one of the polyamide-chlorambucil compounds, and given that the other did not work, it was important to make sure that the concentration of each of the aliquots were similar. Using the spectrophotometer, I found that the while there was a slight range, with 1R being the lowest and 1R-Chl being the highest, they were all acceptably similar. This was not the problem, and I knew finding the appropriate concentrations and the correct buffer would be the next step.

While I was searching for the appropriate buffer for the array experiments, developments in the lab would allow me to revert to the 25mM TEA, 1mM EDTA buffer that was consistent with other published work with chlorambucil and chlorambucil conjugates. This development was the used of 0.01 percent Triton X detergent in the array hybridization mixture.

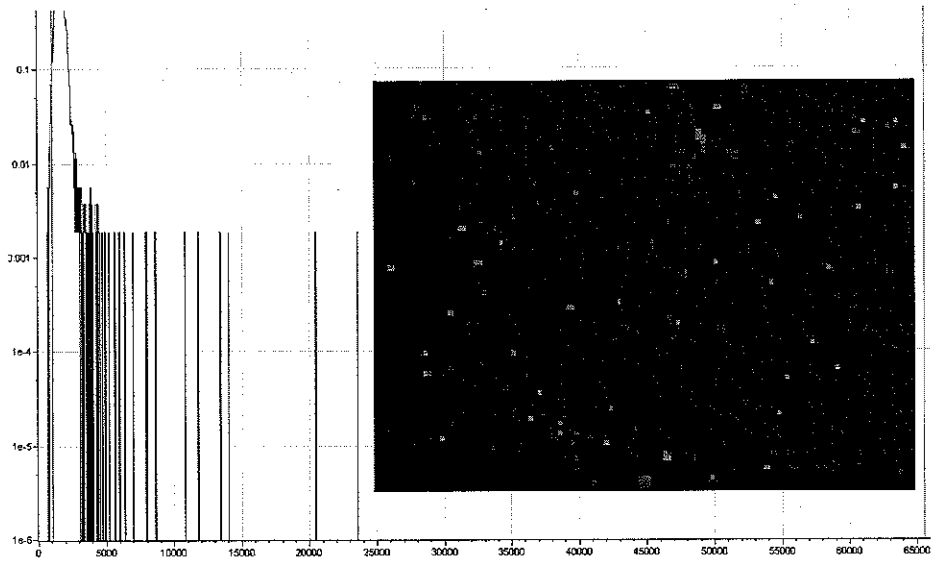
The addition of Triton X detergent allowed for the used of the TEA buffer, which is beneficial because that is the buffer that published experiments with chlorambucil use, and one would imagine that this meant the TEA buffer was the optimal one for chlorambucil's activity. After blocking with milk, I used this buffer to dilute the 1R-Chl polyamide in 5nM concentration and I hybridized the molecule on array CW0053-1 for one hour, as per the usual CSI procedure. When we scanned the array, while it was clear that there were bad spatial patterns and the results were not optimal, I thought I saw some specific binding. Experiments that followed (see figure of array 94-4) would make me reconsider this. Since array CW0053-1 was synthesized in October of 2006 and had been through a lot of experiments, it was possible that the bright features we had associated with 1R-Chl were from another experiment.





5nM 1R-Chl

in CW0053-1



5nM 1R-Chl in CW0053-1, histogram and associated area of array

Around this time, Michelle Farkas finally replied to my email. (Please see Appendix 1 for a copy of her letter. While I asked for suggestions on the buffer, she replied with suggestions regarding the concentration and the hybridization time, but since I had already figured out the buffer this was okay. Her response offered an explanation as to why 8R-Chl seemed to work, whereas 1R-Chl was having a lot of trouble. Farkas said that 8R-Chl was a much more potent alkylator, and that for 1R-Chl, minimal alkylation was evident after four hours at a concentration of 500nM. She said that this concentration was used because that is what the cell culture experiments used, but she was not specific as to what sort of experiment garnered these results. The NAR paper from January of 2007 that reported the unanticipated differences between alpha and gamma turns Furthermore, Farkas suggested that the Cy5 structure may slow the alkylation of the 1R-Chl even further, and quick look at the structure reveals that there are multiple electron pairs that could potentially lend some electronegativity to the molecule, and slightly inhibit the molecule's tendency to react as an electrophile (although I am skeptical as to how extensive this effect may be, due to the fact that the Cy5 moiety and the chlorambucil moiety are on opposite

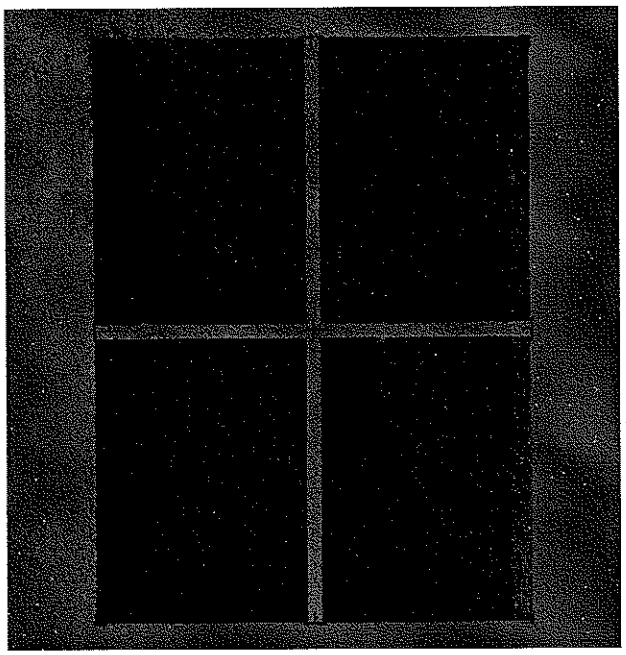
ends of the molecule). Farkas also said that when they do their thermal cleavage to check for alkylation, they let the polyamides equilibrate with DNA for 24 hours.

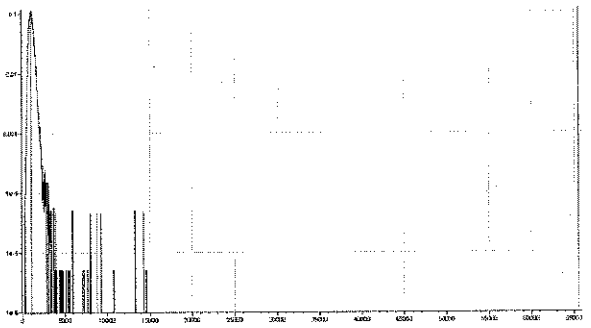
The other comment of note the Farkas made was that depurination is common occurrence in the hybridization of these conjugates, so much so that they use the depurination of alkylated bases to measure the sites and extent of alkylation. Alkylation is supposed to increase with hybridization time, and this is of great concern to me. Balancing the necessary time for alkylation to occur with the likelihood that depurination has not yet occurred is difficult to quantify without numerous experiments. What Farkas recommended was to err on the side of alkylation, and run the experiments for longer to assure that it takes place. The concern I have is that if we plan to understand whether each binding event actually leads to alkylation by washing the array after each hybridization and scanning the array again, we might end up washing some non-covalently bound polyamide that is covalently attached to a depurinated base.

Since it seemed that the 500nM concentration referred to cell experiments, in which the effective concentration was much lower, I decided to keep my concentration at only to 5nM from the previous 1nM with 1R, since 1R had some saturation points even at that low of a concentration, and it would conserve more arrays to go from a lower to higher concentration. The new procedure was:

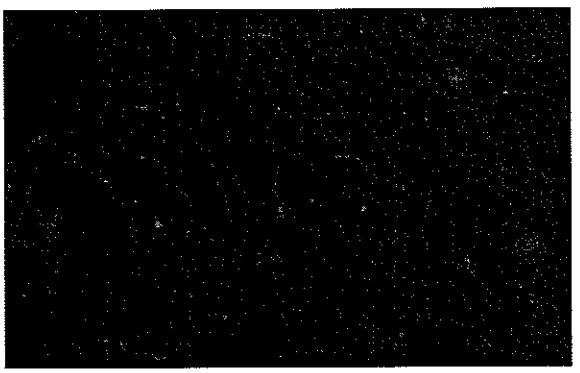
1. Block array with 2.5% milk
2. Hybridize 5nM 1R-Chl/8R-Chl in 25mM TEA, 1mM EDTA, and 0.01% Triton X for 24 hours
3. Scan array
4. Strip array
5. Scan array

With the glimmer of hope that I had from 1R-Chl on array CW0053-1, I used a new array, CW0094-4, to test 1R-Chl at 5nM for 24 hours. The result was that there was absolutely no binding on the features, and there was signal all over the area outside the features☹.

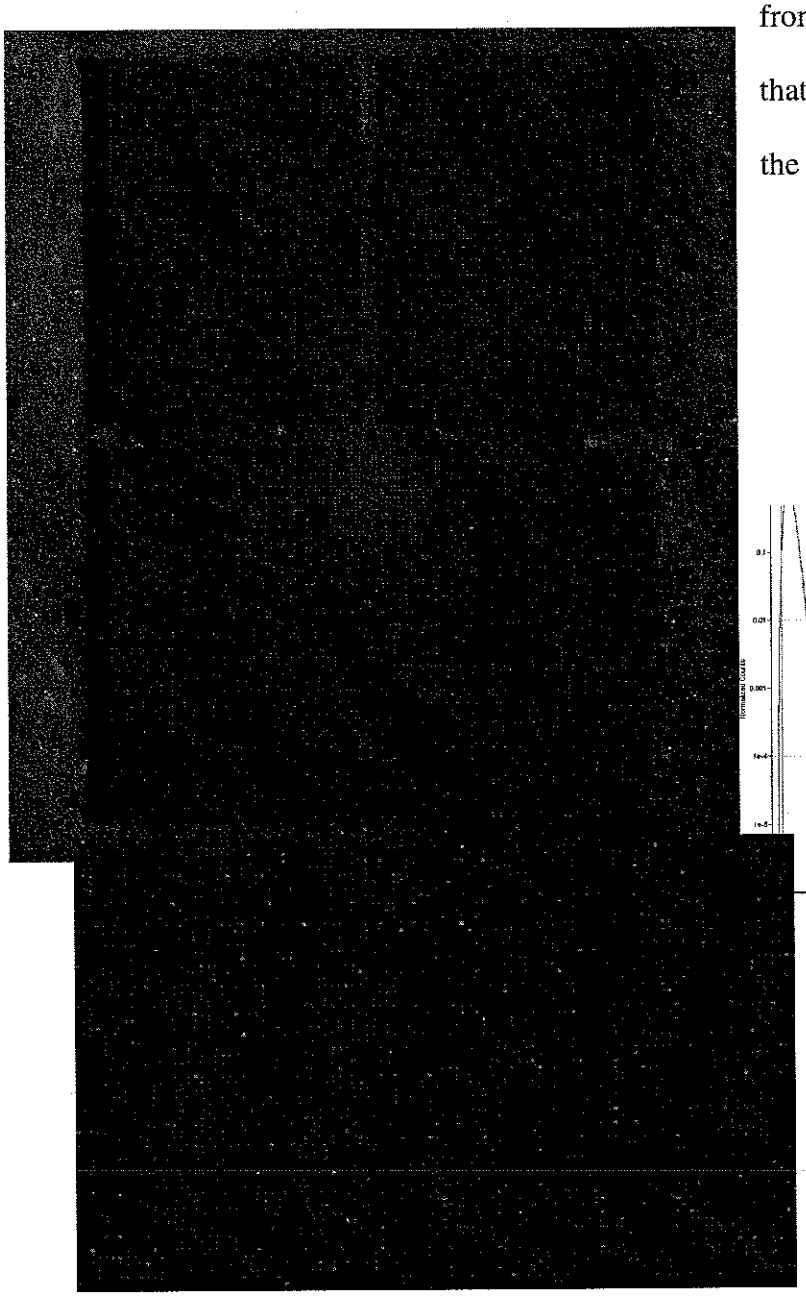




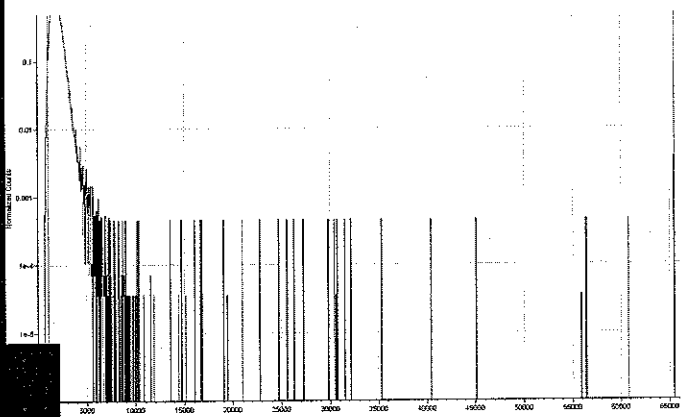
5nM 1R-Chl on array CW0094-4, clockwise from top left: Whole array; histogram; array area of histogram.



This was extremely discouraging. My hypothesis for what happened was that, even with the increase hybridization time, the concentration was too low to bind. This, however, would mean that the binding of the polyamide was dependent on whether the chlorambucil moiety bound and acted. I decided to try 8R-Chl in the same conditions, without even washing the array (because I didn't think it would work…). The results were the opposite of the 1R-Chl results on the same array. The binding was feature-specific, and the ratio between background to signal was between four and five. Also, since I did not strip the array, the background includes the binding from the 1R-Chl experiment. It appeared that the background had doubled from the first to the second experiment.



5nM 8R-Chl onCW0094-4

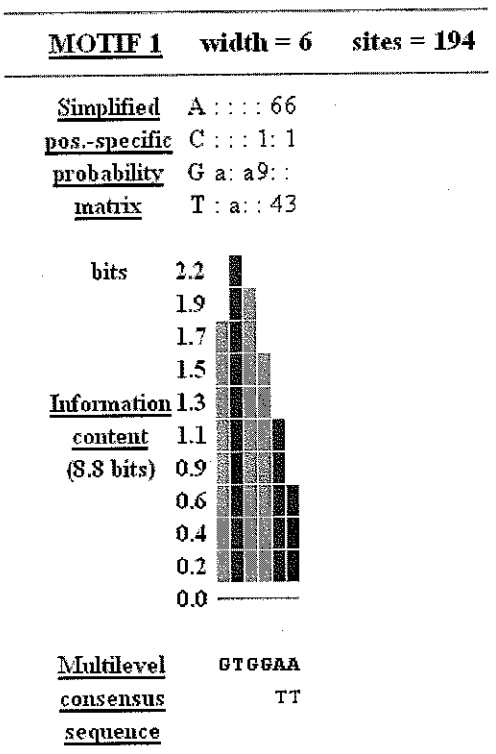


Data Analysis

I have now extracted the data for 8R-Chl, and while MEME was

not able to come up with a consensus sequence, I was able to compare some published data with the extracted data, and it is apparent that the published sequences do have features with high intensities on the array. The sequence I believe fits best is 5'-AGGNGT-3'. However, it should be noted that this data was not optimal, and that this sequence was found to bound both at lower and at higher intensities. Furthermore, the less-specific nature of this binding is consistent with data published on this structure of the PA-Chls, and also with communication with Farkas.

Profiles for 8R and 1R have been obtained, but due to the quality of the arrays, it has been difficult to do the extraction. One attempt to extract the data for 1R showed a sequence that is highly similar to the published data for 1R. What MEME was able to pull out from our data was a sequence 5'-AAGGTG-3' or 5'-TAGGTG-3', whereas the published data notes the consensus sequence as 5'-TAGGTGT-3'. Further work on extracting current data, as well as future experiments to corroborate the findings, will follow.



It is cleat that the substitution of a gamma turn and linker in 8R for the alpha turn and linker in 1R has drastic affects on the ability of the compound to bind, as well as the chlorambucil to alkylate. This is highly consistent with published data on these structures, and, perhaps ironically, gives me confidence in our methods and in the prospects of this project.

Future work

The next step for this project will be in using what we have learned to get a profile for 1R-Chl and to improve the profile that we have for 8R-Chl. First, I plan to use old arrays that have already been used with PA-Chl compounds in order get a good understanding of the time and concentration that 1R-Chl and 8R-Chl need on the array. Then, I will need 6 new 9mer arrays, in two sets (call them A and B) to get the profiles of all the compounds. I will first use set A to get a good profile for 1R and set B to get a profile for 8R. Since these compounds strip fairly well, I will then use set A to get a profile for 8R-Chl and set B to get a profile for 1R-Chl. After (and while) getting these profiles, I will begin working with the data to see where on the mouse genome the target sites seem to appear.