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Synthesis of Novel Porphyrazines and Binding Studies to DNA Quadruplexes

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SYNTHESIS OF NOVEL PORPHYRAZINES AND BINDING STUDIES TO DNA QUADRUPLEXES

A Dissertation

In the Department of Chemistry and Biochemistry submitted to the faculty of the Graduate School of Arts and Science in partial fulfillment of the requirements for the degree of Doctor Of Philosophy in Chemistry at SETON HALL UNIVERSITY by Carlos Ramirez

DECEMBER, 2005
We certify that we have read this thesis and that in our opinion it is adequate in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AFM  Atomic force microscopy
DNA  Deoxyribonucleic acid
TPP  Tetrapyridinoporphyazine
TEPPCI  Tetraethanol pyridino porphyrazine Chloride
TEPPI  Tetraethanol pyridino porphyrazine Iodide
CoTEPP  Cobalt Tetraethanol pyridino porphyrazine Iodide
CuTEPP  Copper Tetraethanol pyridino porphyrazine Iodide
MnTEPP  Manganese Tetraethanol pyridino porphyrazine Iodide
ZnTEPP  Zinc Tetraethanol pyridino porphyrazine Iodide
CuTMPyP  Copper complex of (4-N-methylpyridyl)-porphine
TMPyP2  (2-N-methylpyridyl)-porphine
dA, A  Deoxyadenine
dG, G  Deoxyguanine
dC, C  Deoxycytosine
dT, T  Deoxythymidine
U  Uridine
Guo-3'-P  Guanosine 3'-phosphate
Guo-5'-P  Guanosine 5'-phosphate
CG  Dinucleotide of C and G
ABSTRACT

Widespread interest in the synthesis of porphyrine derivatives has taken a central role in bioorganic chemistry, due to their applications in many areas such as liquid crystals, chemical sensors, and nonlinear optics. The emerging field of photodynamic cancer therapy has prompted an interest in porphyrine derivatives as potential targets molecules for treating cancer and HIV. Tetraacetic porphyrines (Pcs), both metallated and unmetallated have also attracted much attention as photosensitizers for photodynamic cancer therapy.

The synthesis of phthalocyanines from phthaonitride containing metal centers and non-metal centers with organic bases is well known. We have recently reported the synthesis and characterization of a porphyrine and a series of tetraacetic porphyrines, unmetallated and metallated. These porphyrines are tetrapyridino porphyrine.

The unmetallated porphyrines is tetrathiolethrapyridine porphyrinium iodide and the metallated porphyrines are, (Mn, Zn, Cu, Co) = M, M-tetraethiolethrapyridino porphyrinium iodide. The porphyrine and the tetraacetic porphyrines, unmetallated and metallated, were characterized by elemental analysis, ESI-MS, $^1$H-NMR, MALDI-MS, FTIR and UV-VIS. The interaction of porphyrines, tetrathiolethyridino porphyrines (BrTEPP), and its MnTEPP, CoTEPP, CoTEPP, and ZnTEPP derivatives with several nucleic acids have been investigated. In order to understand if porphyrines bind to DNA oligonucleotides, a series of binding studies were
conducted using competitive dialysis, scanning probe microscopy and uv/vis titrations. Also, our interest has focused on the screening of porphyrazines as potential inhibitors of reverse transcriptase of the HIV virus. Our findings indicate that porphyrazines have low binding affinities for single, followed by even lower affinities for double stranded DNA and much greater binding affinities for quadruplex forming oligomers such as TdGdG, GdGdGdG, TdGdT, GdGdGdGTGTGdTdGdG. Our findings also indicate that (HETEPPi), (CaTEPPi), (ZnTEPPi), and (TEPPCl) inhibit the reverse transcriptase enzyme in the HIV virus.
CHAPTER I
INTRODUCTION

Telomeres and telomerase are at the forefront of much nucleic acid research for they hold the key to unraveling how DNA and drugs interact. Telomeres are protein-nucleic acid structures that cap the ends of eukaryotic chromosomes. These terminal structures, composed of repetitive arrays of guanine-rich DNA, such as TTAGGG found in humans, are specific targets for telomere-binding proteins; they play key roles in protecting the chromosome from being destroyed and degraded. Research has also shown that telomere maintenance is an important regulator of cell life span. Activation of telomerase, a dedicated reverse transcriptase that synthesizes telomeric sequences, has been linked with cancer, and research by many groups indicates that telomeres and telomerase perform important functions in both extending and expediting changes in malignant tumors.

The telomeric DNA can reach a length of 15,000 base pairs. Telomeres work by preventing chromosomes from losing base pair sequences at their ends. They also stop chromosomes from connecting to each other. When a cell divides, 25-200 base pairs per division of the telomere is lost. As the telomere becomes shorter, the chromosome reaches a certain length and at this point it can no longer replicate. The cell becomes aged and dies by a process called apoptosis. Telomere activity is controlled by two mechanisms: erosion and addition. Erosion occurs each time a cell divides. Addition is determined by the activity of telomerase RNA subunits that elongates chromosomes by adding in humans TTAGGG sequences to the end of existing chromosomes.
Telomerase is found in fetal issues, adult germ cells and also in tumor cells. Telomerase activity is regulated during growth and has a very small, unchanging activity in somatic cells. Since somatic cells do not usually use telomerase, they age. The result of aging cells is an aging body. When telomerase is activated in a cell, they will continue to grow and divide. This immortal cell theory is important in two areas of research: aging and cancer. Cellular aging, or senescence, is the process by which a cell becomes old and dies. It is due to the shortening of chromosomal telomeres to the point that the chromosome reaches a critical length. Cells are constantly aging. Having the ability to make the body's cells live forever would be the key to a longer life. Basic research on telomerase is the key to unraveling how a cell ages and the process by which it ages. Telomerase research could unlock the path to discoveries related to the aging process, which in turn could help in the process of making new therapeutic agents that target senility.

Cancer cells are malignant cells. The malignant cells multiply until they form a tumor that grows without control. Telomerase has been detected in human cancer cells and is found to be 10-20 times more active than in normal body cells. This provides a selective growth advantage to many types of tumors. If telomerase activity could be stopped, then telomeres in cancer cells would shorten, just like they do in normal body cells. This would stop cancer cells from dividing without control in the early stages of development. If a tumor has already developed, it may be removed and anti-telomerase therapy could be administered to prevent it from causing back. Being able to prevent telomerase from performing its function would change cancer cells from "immortal" to "mortal". Having an understanding of telomeres and telomerase, is the key of discovering
many new drugs and therapies. The future of research in the area of telomerase could reveal insight into how to slow the aging process and destroy cancer. Telomerase enzyme is clearly required in human cells to bypass senescence and crisis responses. During tumor genesis, reactivation of telomerase is likely important in rescuing cells from crisis.

G-quadruplex structures can vary in a number of different ways, including strand stoichiometry and strand orientation, but there is greatest variation at the ends, which if capped by looping structures can consist of any of the four bases in loop sizes of 2 to about 6 bases. The interconversion between double- or single-stranded DNA and G-quadruplex in cells is dependent upon chaperone proteins, such as the subunits of the telomere binding protein from Oxytricha that facilitate G-quadruplex formation, as well as upon helicases such as Sgs1 from yeast, which resolve these structures. Proteins such as RAP-1 are also known that bind to and stabilize these G-quadruplex structures.

The facile interconversion, at physiological conditions, between double- or single-stranded DNA and G-quadruplex structures, together with the known occurrence of binding or chaperone proteins, makes these secondary DNA structures attractive candidates for biological signaling molecules, based upon their unique molecular recognition properties in contrast to duplex DNA. In many respects, the folding of single-stranded DNA into G-quadruplex structures is analogous to the folding of peptides into proteins, as the primary base sequence is the main determinant of the folded G-quadruplex structure. When the genetic information encoded by DNA must be passed on to the next generation, a mechanism for interconversion between secondary DNA and duplex DNA structures must be available. This requirement for interconversion between
the different forms of DNA points to a potential weakness in the system, as secondary "trapped" DNA structures might disrupt signaling mechanisms or prevent replication.31

The original purpose for the design of G-quadruplex-active molecules was inhibition of telomerase by sequestration of the single-stranded DNA primer as a G-quadruplex structure, thus eliminating the substrate required for the reverse transcriptase activity of telomerase.32 This approach to inhibition of telomerase is in contrast to that involving catalytic inhibition of the enzyme. Both approaches to inhibition of telomerase have inherent problems. The selectivity imparted by the absence of a requirement for telomerase from all but germ-line cells in normal tissues gives rise to the anticipated selective toxicity to cancer cells that uniquely require this activity for telomere maintenance and survival; however, the prolonged time for sufficient telomere erosion is potentially a major therapeutic problem.33 In contrast, telomere disruption by stabilization of G-quadruplex structures lacks the selectivity associated with the unique requirement for telomerase in cancer cells and depends instead upon whether or not telomere disruption is more detrimental to cancer cells than to normal cells. As yet, data are lacking that would define such differences, although it is likely that the onset of events triggered by telomere disruption is much faster than those mediated by telomerase inhibition. So, strategies that depend purely upon disruption of telomeres lack a solid rationale for selective therapeutics for cancer. Nevertheless, encouraging data are emerging that shows that despite a lack of significant telomere shortening, which would result from the inhibition of telomerase, telomeric disruption, presumably through trapping of G-quadruplex structures, results in short-term biological effects such as formation of anaphase bridges, apoptosis, and cell death.34 A recent report from the
Gedner research laboratory has demonstrated that it is the shortest telomere, not average telomere length, that determines chromosomal stability and cell viability. Cancer cells with just one abnormally short telomere will presumably be made more sensitive than normal cells to catalytic inhibitors of telomerase. They may also be more sensitive to G-quadruplex-interactive compounds if telomere modification by these agents leads to degradation of the telomere.

G-quadruplex-forming sequences are also found in a number of transcriptional regulatory regions of important oncogenes, including c-myc, c-myb, c-fos and c-abl. Because of the polyuridine-polypyrimidine nature of these duplex sequences, which contain four or more runs of clusters of three or more guanines on the purine-rich strand, they often show a single-stranded character and hence are nuclease hypersensitivity regions. In the c-myc promoter, the purine- and pyrimidine-rich strands bind transcription factors (CBP and lnRNP) required for transcriptional activation. As these elements can also form G-quadruplex and i-motif structures, it is possible that these secondary DNA structures inactivate transcription, and their conversion to the duplex region is required for transcriptional activation. G-quadruplex-stabilizing molecules that would prevent this transition would therefore inactivate c-myc expression. As down-regulation of c-myc expression in tumor cells by only 30% leads to a dramatic reduction to ras and raf transformation, the selective stabilization of G-quadruplex structures in the promoter regions of c-myc should lead to selective effects on cancer cells.

Our group has been investigating the synthesis and binding of metallic and nonmetallic cationic porphyrines, especially the nonmetalated tetraethanol-pyridino
porphyrazine iodide, (TEPPi), and metalated tetraethanol-pyridine porphyrazines iodide having Mn, Co, Cu, and Zn as the center metals (MTEPPi) with polymorphic DNA structures. Physical properties of these porphyrazines such as molecular dimensions, presence of a chromophore, outside positive charges and water solubility make them favorable for intercalating into, or stacking, the major or minor groove or by electrostatic forces with multistranded structures. Interactions of these porphyrazines with multistranded DNA have been explored, especially the binding modes via UV/VIS studies, competitive dialysis studies, competitive binding studies with ethidium bromide, AFM studies, and specifically their utility as Reverse Transcriptase inhibitors in HIV. This research has shown that porphyrazines have no binding affinity for single stranded DNA, and that there is more affinity towards DNA quadruplexes than DNA duplexes. It has also been demonstrated that TEPPi and MTEPPi porphyrazines are able to inhibit Reverse transcriptase RNase and reverse transcriptase polymerase.

Initially, we became interested in synthesizing model compounds that would interact with different polymorphic forms of DNA including self-assembling quadruplexes and to exploit their activity as HIV inhibitors. After three years, we were able to find facile and novel routes to the synthesis of nonmetalated and metalated porphyrazines. After fully characterizing these porphyrazines, our next task was to determine if these molecules bind to polymorphic DNA structures and to demonstrate their utility as potential therapeutic agents for HIV.

The binding studies and competitive dialysis studies were monitored by visible absorption spectroscopy. Competitive binding studies with ethidium bromide were monitored by atomic force microscopy. The utility of these porphyrazines as potential
HIV inhibitors was done at Carnegie Mellon Institute of Viral Diseases under the direction of Dr Mike Pamiak.

It must be noted that the main thrust of this project was to synthesize novel porphyrin complexes and to demonstrate if these porphyrin complexes can selectively recognize and stabilize, single, double or multistranded DNA quadruplexes, which in turn could inhibit telomerase activity. Hence, the focus of this research was to investigate if these porphyrin complexes would bind and selectively recognize multistranded DNA. This effort was to further our understanding of how anticancer drugs specifically target telomeric DNA and to find their utility for other diseases.

This dissertation is organized in the following order: an introduction is given in Chapter 1; an historical overview is given in Chapter 2; the theory, materials and methods are given in Chapter 3; results and discussion are given in Chapter 4; and Chapter 5 is comprised of the conclusion and other possible future work, followed by references.
CHAPTER II
HISTORICAL

DNA, is a nucleic acid that carries the genetic information in the cell and is capable of self replication and synthesis of RNA. It is composed of a series of monomer units that are linked through covalent bonds. Each of these monomer units, called nucleotides, are made of a 2'-deoxyribose sugar connected with a phosphate group at its 5'-carbon and a purine or pyrimidine base at its 1'-carbon. The bond between two successive nucleotides is through the phosphate group on the 5'-carbon of one unit and the hydroxyl group on 3'-carbon of the next one, named the phosphodiester linkage. In this manner, the construction of long nucleic acid chains is established. Figure 1 illustrates the chemical structure of a small DNA polymer chain by displaying the arrangements of the deoxyribose phosphate backbone and sugar molecules along with the four major bases of DNA.

Watson and Crick were the first to propose the acceptable DNA structure in 1953 using X-ray diffraction fiber studies. Along with the X-ray diffraction data, earlier chemical data obtained from other research groups helped to confirm the proposed structure. According to their model, DNA is a two-stranded entity where the strands interwine to form a right-handed double helix. The strands run anti-parallel to each other, joined together through interstrand hydrogen bonds between purine and pyrimidine bases. The base pairing is A-T and G-C in which adenine and thymine are bonded by two hydrogen bonds and guanine and cytosine are bonded by three hydrogen bonds.
respectively, as depicted in Figure 2. These matching base pairs can stack on one another with their planes perpendicular to the helical axis.
Figure 1: A single strand of DNA of four nucleotide residues [Sinden, 1994].

Figure 2: Formation of DNA duplex from Watson-Crick base-pairing for CG (left), TA (center), and Hoogsteen base pairing for AT (right) [Blackburn and Gait, 1996].
Studies of DNA fibers using X-ray diffraction helped to elucidate two types of helices that configure into two different forms, A-DNA (Figure 3) and B-DNA (Figure 4). Both of these conformations are oriented as right-handed helices, the A-DNA conforms under the conditions of low humidity while the B-DNA conforms under the conditions of high humidity. Figure 3 and 4 illustrate B-DNA with well-defined major and minor grooves along its axis, where the A-DNA has both grooves of nearly equal in depth. Although the majority of DNA occurs in the B-form, others such RNA-RNA and DNA-RNA helices exist in A-form.

These structure conformation differences are influenced by the solvent conditions at which the DNA or RNA resides. In aqueous solutions, there is extensive hydrogen bonding between solvent water molecules and DNA. These are established by the multiple hydrogen bond donor and acceptor sites present on sugars, phosphates and bases. The phosphate group is a strong acid with a pKₐ of about 1 while other residues in a DNA molecule carry a negative charge at physiological pH.
Figure 3: A-DNA double helix [Mathews and van Holde, 1990]
Figure 4: B-DNA double helix [Mathews and van Holde, 1990]
I. Telomere and Telomerase

Telomeres are the nucleic acid-protein complexes that occur at the end of eukaryotic chromosomes. Telomeric DNA has been characterized to contain tandem repeats of guanine rich or G-tract sequences such as d(TGGGG) in *Tetrahymena*, d(TTTGGGG) in *Oxytricha*, and d(TTGGG) in *Homosapiens*. It constitutes the terminal 10kb of all human chromosomes. Telomeres’ functions are to maintain the stability and integrity of chromosome, preventing incomplete replication and end-to-end fusions.  Without the telomeric repair mechanism, the telomere length maintenance will not suffice in preventing gradual loss of genetic information since telomeres of most human cells shorten with each cell division due to incomplete replication of linear DNA. This limits the proliferative life span of most human somatic cells to between 50-80 cell divisions before they enter a state of replicative senescence, termed as M1. On the other hand, telomer lengths of immortalized cells are constantly maintained and tightly regulated by enzymatic mechanism of telomerase, which specializes in reverse transcriptase. The mechanism involves adding telomer repeats onto the pre-existing 3' single stranded overhangs of telomeric DNA. Figure 5 demonstrates the mechanism of how telomerase maintains the telomere length in eukaryotes.

Telomerase is a ribonucleoprotein complex that consists of various subunits such as the catalytic reverse transcriptase subunit (hTERT) and an RNA component (hTR), and a number of other associated protein subunits. Telomerase activity has been detected in 85-90% of human tumors and tumor-derived cell lines, but is largely absent in most of...
Many researchers have proposed that selective target of telomerase is an avenue for designing new anticancer drugs.

Figure 5: Mechanism by which telomerase adds short telomere segments to the 3' end of chromosomal DNA. These segments, which can then be primed, allow 5' → 3' synthesis on the lagging strand.
Research from the literature indicates the development of chemothterapeutic agents that are capable of providing selective inhibition of human telomerase for the treatment of cancer. The evolving knowledge on the composition, function and roles of telomerase and telomeres in cell immortality and cancer has prompted the development of numerous distinct rationales for the development of various inhibitors. Among different strategies are antisense-based oligonucleotide inhibitors targeted against the template region of hTR, traditional reverse transcriptase inhibitors, and agents capable of promoting and/or stabilizing high-order DNA quadruplex formation.

The hTR transcript comprises approximately 450 nucleotides and contains an 11-nucleotide sequence (5'–CUAACCCUAAC) template region complementary to the human telomeric repeat sequence 5' d(TTAGGG). The active functional part of the RNA is divided into two distinct domains. One of the active domains consists the 3' end that functions as an alignment for binding of the substrate, while the 5' –domain acts as a template for telomere elongation. The intrinsic accessibility of the RNA template region of hTR offers an attractive target for enzyme inhibition compared to other antisense-directed mRNA targets due to its necessity for binding telomere ends.

Since telomerase is a highly specialized DNA polymerase with reverse transcriptase activity, its function is to synthesize telomeric repeats for attachment to the 3' tail-end of the telomeric DNA strand using an RNA template. It has been proposed that the enzyme activity may be inhibited or prevented by chain terminating deoxynucleoside triphosphates (ddNTPs) and other nucleoside triphosphate reverse transcriptase inhibitors.
There is considerable interest in the development of highly selective and potent small-molecule non-nucleoside inhibitors of telomerase. In this regard, several distinct rationales have been explored. These include ligand-type agents, which are capable of promoting the formation and/or stabilization of high-order DNA quadruplex structures. In addition, the effects of established anticancer agents on telomerase activity have been examined in an attempt to determine if telomerase inhibition is a mechanistic component of drug efficacy.65

The observation of tandemly repeated segments of G-rich bases found in telomeric DNA from most eukaryotes prompted the conclusion that telomeres must serve some distinct biological roles.66 In addition, the discovery of DNA quadruplex formation from such sequences has stimulated extensive development on the evaluation of quadruplex structure and function during the past decades.67 These structures are believed to play a vital cellular role, despite their lack of detection in vivo, and the conservation of telomeric DNA may be related to their inherent quadruplex formation.68 It has been established that RNA template region of telomerase requires a linear unfolded telomeric primer to affect telomere elongation; thus the presence of telomeric quadruplex structure leads to telomerase inhibition.69 As a result, DNA quadruplexes can be viable targets for the development of telomerase inhibitors. DNA quadruplexes inhibit the enzymatic functions of telomerase by preventing accessibility to the linear DNA substrate. This functional cue has prompted many researchers to explore new ligands or related agents that can selectively stabilize DNA quadruplexes and hence mimic K+ ions as telomerase inhibitors.70
II. Polymorphic Nature of DNA

Most of the DNA in cells occurs as one of the three secondary structures - random coil, B form, or A form. But this does not extensively cover other conformational possibilities for this extraordinary molecule. In recent years, several other exotic structures have been observed and discovered by many researchers in various scientific fields. The types of DNA secondary structures were found to be very dependent upon the DNA sequence as well as the solution conditions.\(^7\)

One notable alternate structure of DNA is Z-form, which is a left handed double helix (Figure 6). The crystal structure of Z-DNA was first characterized using X-ray diffraction analysis to evaluate the dimer with sequence d(CpGpCpGpCpG).\(^7\) It has been established that sequences with alternating dCdG are the most adept at forming Z-DNA under high salt conditions. It has also been demonstrated that both B and Z forms could exist in the same DNA molecule simultaneously, separated by a small junction.\(^7\)
Figure 6: Z-DNA double helix [Mathews and van Holde, 1990]
Another alternate helical structure is triple-stranded DNA, first observed in 1957.\textsuperscript{74} The phenomenon was formed by the binding poly(dCT) to poly(dGA)•polyd(CT) and poly(dG) to poly(dG)•polyd(C) by non-Watson-Crick base-pairing. The formation of three stranded structures entails the binding of oligonucleotides in the major groove of B-horm DNA by forming Hoogsteen or reversed Hoogsteen hydrogen bonds using the N-7 of the purine bases of the Watson-Crick base-pairing (Figure 7). In theory, G can form a base-triple with a G•C pair and A with a T•A but the only combinations that have isomorphous location of their C-1' atoms are the two triplets TxA•T and C'xG•G (C' is the N-3 protonated form of cytosine). This means that the three strands of triple-helical DNA are normally two homopyrimidines and one homopurine.\textsuperscript{75}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Formation of DNA triplex from Non-Watson-Crick base-pairing for TAT (left), TAA (center), and CGG (right) from Hoogsteen base pairing.}
\end{figure}

Other structures, such as cuciform DNA structures, have been shown to form within supercoiled DNA \textit{in vitro}\textsuperscript{76} and have been proposed to form \textit{in vivo}.\textsuperscript{77} Holiday junctions have been reported to form during the recombination of two pieces of double-
stranded B-DNA in vivo. As mentioned earlier, three-stranded helical DNA has been observed and studied. Also, the existence of four-stranded DNA (e.g. quadruplex, tetraplex, or G-tetrad) has been observed from sequences rich in guanine bases.

III. DNA Quadruplexes

Guanine tetrads were first discovered in 1962 when Gellert et al. reported the aggregation of guanine mononucleotide into gels. Gellert proposed that the formation of a four-stranded structure was from the association of the guanine bases into a quartet structure through hydrogen bonding. This guanine-quartet structure is connected by hydrogen bonding of each guanine to two other guanines to form a closed ring with a four-fold rotation axis, as depicted in Figure 8.

Other researchers reported the formation of Poly(G) into self-associated complexes with extremely high thermal stability. The CD and IR spectra of poly(G) are very similar to those of guanosine gels. This suggests that poly(G) might also be forming a four-stranded structure. This hypothesis was later confirmed by two X-ray fiber diffraction studies. Based on the detail evaluation of the diffraction patterns of poly (G), the formation of four-stranded helical structures was positively confirmed. It was proposed that the guanines could be forming Hoogsteen type hydrogen bonding similar to those found in G/G mismatches. From fiber diffraction studies of poly-G helices, they found that the four-stranded helical structure oriented with a 3.4 x 10^-10 m rise between hydrogen bonded guanine quartets as indicated in Figure 9.
Figure 8. Guanine-quartet (G-Tetrad) hydrogen-bonding array (R-H for guanine and R-phospho-ribose for nucleic acids) (Gellert, 1962).
Figure 9: Cyclic array of four guanine bases (a); Quadruplet Helical Structure (b); G₄T₄G₄ in Na⁺ solution determined by NMR (c); G₄T₄G₄ in K⁺ solution determined by X-ray Crystallography (d) [Williamson, 1994]

Furthermore, Sen and Gilbert also studied several oligomers containing G-rich clusters corresponding to the immunoglobulin G switch regions that constitute the sites for recombination between the variable and the constant sequences during the differentiation of B lymphocytes to plasma cells. It was found that the Watson-Crick base-paired DNA forms structures upon incubation in Tris-HCl buffer at 4°C, in which the C-complementary strands are looped out to form four-stranded DNA complexes ("M" strands) composed of base-paired guanine "quartets". They referred to these complexes as "G-4 DNA". They extended these studies to assess the effects of monovalent cations.
on the structures formed by telomeric DNA.\textsuperscript{88} They observed that in the presence of Na\textsuperscript{+}, the complexes contain four parallel strands and that the formation of the complex is a second order process. This suggests that the rate-limiting step in quadruplex formation involves dimerization of strands to form an intermediate duplex structure(s). Increasing K\textsuperscript{+} initially stimulates and then blocks the formation of G-4 DNA. As the K\textsuperscript{+} is increased above 50mM, their results show that intermolecular "fold-back" structures become predominant. Sen and Gilbert proposed novel DNA superstructures formed by telomere-like oligomers.\textsuperscript{89} They found that oligomer T\textsubscript{4}G\textsubscript{4}T forms a unique G4-DNA product at neutral pH in the presence of Na\textsuperscript{+}, K\textsuperscript{+}, or Rb\textsuperscript{+}; however its isomeric counterpart T\textsubscript{4}G\textsubscript{4} in K\textsuperscript{+} or Rb\textsuperscript{+} generates an additional ladder of products of substantially lower gel mobility. They demonstrated that these larger complexes contain, respectively, 8, 12, or 16 distinct strands of oligomers. Methylation protection experiments suggested a nested head-to-tail superstructure containing two quadruplexes bonded front-to-back via G quartets formed by out-of-register guanines. The results of this work are schematically shown in Figures 10, 11, and 12.

In order to understand the stability of the G-tetrad, chemical probes have been used extensively. The guanine base in a G-tetrad is nearly saturated with hydrogen bonds, and hence is sensitive to small changes in the environment. DNA from Otgericha has determined to be resistant to both endo- and exonucleases. The N-7 of the guanine bases are involved in hydrogen bonding with neighboring bases and this affords stability from compounds like dimethylsulfate. Dimethylsulfate can methylate the N-7 of guanine and cause depurination of DNA. In fact, the stability of the chromosomal telomerase is due to the non-exposure of N-7 of guanine to external chemical agents. For example,
several anticancer compounds such as cisplatin target the N-7 of guanine in double stranded DNA.\textsuperscript{90}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Schematic of a back-to-back arrangement of a G-8 DNA complex formed from a G-4 DNA.}
\end{figure}

Other studies on the telomeric DNA complexes that contained 3'-'dT(GG)\textsubscript{2} overhanging DNA showed that the molecules dimerize in a Na\textsuperscript{+} or K\textsuperscript{+} in a dependent manner in which all N7 atoms of the guanine bases of the complexes are inaccessible to DMS modification.\textsuperscript{91} The G-tetrad complexes are stable in buffers containing greater than 100 mM KCl. The authors also suggest that the thymidine residues may act as "spacers" that serve with two function: (i) to reduce steric clash between the quadruplex and adjacent duplex regions and (ii) to allow G-DNA hairpin formation by acting as loops. This is consistent with the affinities of the bases for water, since T is the most hydrophilic and G the most hydrophobic of the DNA bases.\textsuperscript{92}
Figure 11. Schematic of back-to-front (top) and back-to-back (bottom) models for G-8 DNA which involve bonding of two G-4 DNA units via quartet formation by slipped guanines from either unit. The un-bonded G's are circled.

Figure 12. Schematics for G4-DNA formed by TcG3 (A) and TcG7 (B) and the sugarcane type model of G8-DNA formed by TcGn (C).

Recent studies have reported the architecture and stability of these quadruplexes to be highly dependent on DNA sequence as well as the conditions (e.g., buffer, ionic strength, cations present, and temperature) under which the DNA is prepared. A number
of structural and thermodynamic studies on different G-DNA molecules have revealed that cations induce and stabilize quadruplex formation in the order $\text{Cs}^+<\text{Rb}^+<\text{K}^+<\text{Na}^+<\text{Li}^+$ for monovalent cations and $\text{K}^+<\text{Ca}^{2+}<\text{Mg}^{2+}$ for divalent cations. This indicates that $\text{K}^+$ has the optimal ionic radius for complex formation and stabilization. The effect of different cations on the quadruplex stability is illustrated in Figure 13.
Williamson et al., studied the effects of monovalent cations on DNA oligomers corresponding to the four repeats of telomeric sequences of *Oxytricha* and *Tetrahymena* using non-denaturing gel electrophoresis experiments and DMS protection assay. They found that these DNAs migrate faster than those of the corresponding single-strands under native conditions and that all N-7 positions of guanine residues were resistant to DMS modification in the presence of 50 mM NaCl, KCl, and CaCl. UV cross-linking of the thymine residues in the presence of Na\(^+\) is concentration independent, thus indicating an intramolecular reaction. They concluded that the oligomers consisted of four repeats of telomeric sequences forming a compact, intramolecular folded back G-quartet structure, in which the strands are pair-wise antiparallel and two of the four guanines are in a syn conformation. They also noted that an arrangement of the four guanines with Hoogsteen pairing is necessary to form G-quartet in a syn/syn/syn/anti alternating with
respect to the glycosidic bonds (Figure 9). Such an arrangement allows for the alkali cations K⁺, Na⁺, and Cs⁺, but not Li⁺, to form bonds with the four keto groups within the central cavity.⁹⁴

The molecular modeling on the interaction between quadruplex structures with different mono- and divalent ions was determined by force field calculation in both vacuum and solution. It was deduced that cations like K⁺ are tightly sandwiched between both top and bottom neighboring planar of the G-tetrad units build from the guanine bases of the four DNA strands (Figure 14). The calculations in solution show that the position of water molecules in close proximity to the DNA channel has a strong influence on the interaction potential, and hence on the capability of the cations for leaving and re-entering its potential sites.⁹⁵ It was shown that van der Waals interactions at short distances determine the specific characteristics of the different cations.
Nuclear Magnetic Resonance (NMR) studies have also been used to characterize detailed structural information about G-tetrads. Due to the repetitive nature of telomeric sequences, the NMR analysis is spectrally overwhelming. Therefore, the assignment of resonance on the NMR spectrum becomes very difficult. These types of problems can be reduced using two-fold or four-fold symmetric structures that simplify the NMR spectrum for evaluation. The first detailed study on G-tetrads was done on G3T4G2 by
revealing the glycosidic torsion angle alternated syn-anti for each adjacent pair of G's along the strand.96

A more detailed study was later reported on the sequence G₄T₄G₄. In the presence of 50 mM Na⁺, a single predominant species is formed by this oligomer even at 4 mM DNA strand concentration. Eight imino protons were observed, one for each of the guanines, indicating that each guanine is involved in hydrogen bonding. As observed for G₂T₂G₂ the glycosidic torsion angles alternate syn-anti-syn-anti along the strand.97

Investigations on telomeric DNA sequence d(T₄G₄) from Oxytricha through NMR have also been reported in which a single copy of the sequence produces an exceptionally stable parallel stranded tetramer consisting of four layers of G-quartets in an orderly helical array.98

They proposed that all the G's in this right-handed helical quadruplex are in C2'-endo, anti configuration and the T4 adjacent to the G-layer, shows the right-handed helical structure similar to that of the G-tract and the rest of the T-tract was more flexible as shown in Figure 14.

The DNA aptamer d(GGTTGGTGGTGGTGG) has also been studied by NMR. This particular DNA exhibits a highly compact and symmetrical structure, which consists of two tetrad of guanosine base pairs and three loops. The residues of the tetrad alternate anti/syn/anti/syn as depicted in Figure 16.99

In addition, early circular dichroism study on G₄T₄G₄ oligomers indicate a four stranded quadruplex when x =1, a quadruplex formed from the association of two fold back structures when x = 3 or 4 and a mixture of two possibilities when x = 2.100

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Recent NMR studies have shown that the preferred conformation for G₄T₄C₄ consists of the two-stranded structure. The exact orientation of the T₄ loop is still under debate. Figure 15 displays some of the possible motifs generated from different DNA oligomers.

Figure 15. (A) Energy minimized quadruplexed structure of the sequence dT₄G₄. Eight planes containing four G's and four T's. (B) Top view of the stacking at the T₄-G₅ steps. Each T stacks partially below two G-bases in the G₅ layer, in an interaction that seems to be stabilized by a K⁺ ion located between the planes.
Figure 16. (top) A depiction of the tertiary structure of the aptamer is shown. The dashed lines indicate the location of the G-tetrad. (bottom) A depiction of a syn-anti-syn-anti tetrad is shown.
Figure 17. Structure of parallel stranded quadruplex T₆G₄(A), hairpin C₃T₆G₆T₆G₃(B) and bimolecular quadruplex G₃T₆G₄(C).

Figure 18. Schematic representation of the bridging of successive quadruplexes by two strands, resulting in a linear superstructure with the individual tetrads and the periodic “pockets”.

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Recent studies have demonstrated that certain G-rich sequences can self-assemble, under certain ionic conditions, to give rise to higher order structures. Studies from our laboratory showed that G-rich DNA such as C5T4G4T4G4 and G4T4G4 could form higher order structures as shown in Figure 18 studies, and gel electrophoresis experiments suggested that these oligomers were unable to self-assemble under Na⁺, K⁺ or Mg²⁺ alone, but would self-assemble into multistranded species of high molecular weight in the presence of 100 mM K⁺ plus 20 mM Mg²⁺. Since higher order structures are not observed with K⁺ or Mg²⁺ alone, these cations must behave in a synergistic manner in order to stabilize the self-assembly of supermolecular structures.

It has been reported that similar type of self-assembly using G5T4G4 in the presence of 50 mM Na⁺, or K⁺ as shown in Figure 16. The self-assembly process was more efficient in the presence of Na⁺ plus Mg²⁺ or K⁺ plus Mg²⁺. The authors also came up with similar structure as proposed by our group. They also studied the self-assembled "G-wires" through atomic force microscopy (AFM). They found that the "G-wires" formed in the presence of Na⁺/Mg²⁺ or K⁺/Mg²⁺ were much longer than the ones formed under these cations alone (Figure 18). They also observed that these quadruplexed DNA wires are more rigid as compared to the duplex DNA. Thus the self-assembly of oligomers possessing G1T2G4 segments (where x = 1-4 or higher) generates a linear scaffolding with periodic spaced pockets (Figure 19).
In addition to electrostatic binding sites on the DNA backbone these periodic "pockets" also possess hydrophobic and hydrogen binding sites, which are ideal candidates for binding of small ligands within these pockets. Ligand binding studies by our laboratory have been evaluated to probe these types of structures (Narayanan A., Ph.D. thesis, 1997). \(^{111}\)

Currently work is being done by our laboratory to investigate the effects of sequence and environmental conditions on the self-assembly of these superstructures. By understanding the properties and behavior of the self-assembly process, we may able to engineer or "tune" these superstructures into relevant candidates for biological and nanotechnological applications. Thus, specific designed DNA oligomers with sequences
similar to the naturally occurring repeats mentioned above might provide a simplified model for evaluating the structural and thermodynamic properties of quadruplex DNA formation.

Figure 20. Schematic representation of the pocket created in the self-assembly of DNA oligomers possessing G$_4$T$_3$G$_4$. 
IV The origin of Phthalocyanines

Phthalocyanine was accidentally discovered as bluish stable impurities in a reactor made of iron in 1928 that was used to manufacturer phthalimides. The word "phthalocyanine" was made from Greek by Linseid in 1934.\textsuperscript{112}

To date, phthalocyanine, metal phthalocyanine and metal phthalocyanine derivatives have been manufactured in large quantities, and used as dye stuffs and pigments, because of their blue to green coloring and stability to visible light.

Molecular structures of phthalocyanine, metal phthalocyanine and metal phthalocyanine are similar to the naturally occurring porphyrins which were known to make up vitamin B\textsubscript{12}, chlorophyll and hemoglobin. Porphyrins are macrocyclic molecules composed of four pyrrole units, while phthalocyanines consist of 4 isoindole units with nitrogen atoms at each meso position. Phthalocyanines are also called tetrabenzoazaporphyrines or tetrabenzoazoporphyrines.\textsuperscript{113}

In this decade metal phthalocyanines and their derivatives are known for their use in functional chromophores for electronics, sensors, biology and pharmaceuticals because they possess a molecular structure similar to porphyrins which have exhibited high electron transfer abilities owing to their \(\pi\) electron conjugated ring system.\textsuperscript{114}

Phthalocyanines containing central metal atoms are called metal phthalocyanines. Metal phthalocyanine have been prepared from every group in the Periodic Table.\textsuperscript{115}

Phthalocyanines, metal phthalocyanines and metal phthalocyanine derivatives can be synthesized by various methods. Phthalocyanine is synthesized directly from diiminoisoindoline or phthalonitrile (1,2-dicyanobenzene) by cyclotetramerization.

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Phthalocyanine, metal phthalocyanine derivatives are also prepared from diiminisoindoline in the presence of metal salts. In another major method, phthalonitrile is used as a precursor in the presence of metal chloride. This method is called Linstead's method or the Phthalonitrile method.\textsuperscript{116}

Another major route for the synthesis of phthalocyanines uses phthalic anhydride, a metal salt, urea and a catalyst. This method is widely used in the manufacturing process, and is called Wyler's method. Most metal phthalocyanines derivatives are produced by the reaction between unmetallated phthalocyanine or lithium phthalocyanine in the presence of a metal salt.\textsuperscript{117}

V. Telomeres and cancer

Telomeres give our cells finite life spans. How is it that cancer cells seem to possess infinite life spans? How can they reproduce and spread infinitely? How do cancer cells get around the limits telomeres impose on our healthy cells and thereby become immortal?\textsuperscript{114}

In most organisms, including humans, the enzyme telomerase can add new repeats of the TTAGGG sequence onto the tips of telomeres to lengthen them. In most non-human organisms, telomerase is always active when cells are dividing. This makes up for the gradual telomere shortening from replication and cell division. This activity, regulated by certain proteins, keeps telomere length more or less constant.\textsuperscript{119}

Human cells are different. In most of them, telomerase is turned off. This means telomeres shorten as cells continue to divide. However, almost 90% of all cancer cells
have been found to possess telomerase. Telomerase is an enzyme that "rewinds" our mitotic or cellular clocks.\textsuperscript{120} Telomerase strengthens and lengthens our shortened telomeres, replacing the bits of DNA lost in ordinary cell division. If telomerase stops telomere shortening, then in theory, those cells with telomerase can live forever. Since most cancer cells contain telomerase, researchers believe it is a critical factor in conferring immortality upon these cells.\textsuperscript{121}

Inactivation of telomerase and the resulting telomere shortening likely evolved in organisms to reduce the incidence of cancer. By causing replicative senescence, telomere shortening acts as a road block to the abnormally high amount of proliferation associated with the development of cancer.\textsuperscript{122}

However, telomere shortening itself may be an active contributor to the genetic abnormalities that trigger cancer. There is growing evidence of an association between short telomeres and a greater risk of cancer development in humans. In addition, laboratory evidence has shown that short telomeres may contribute directly to the progression of the earliest stages of certain cancers. In an experiment involving cells taken from prostate cancer patients, researchers from the Johns Hopkins University School of Medicine found that telomeres in cells from precancerous lesions were four times shorter than telomeres in cells taken from surrounding normal tissue.\textsuperscript{123} The researchers believe that short telomeres contribute to cancer due to increased genomic instability within cells with short telomeres.\textsuperscript{124}
VI. DNA-Drug interactions

Drugs are simply small molecules with biological activity. These can be used as therapeutic agents in medicine, or also as tools in research laboratories. There are two methods by which these small molecule drugs can interact with the DNA double helix. They can bind in the grooves or alternatively, they can stack in between the base pairs within the helix (called "intercalating").

I. Intercalating Drugs

This group of drugs tend to be planar molecules, primarily aromatic rings. This is necessary, first for the steric features of the double helix, and also for the hydrophobic quality of the nitrogenous bases. These drugs cause drastic local changes in the geometry of the DNA where it interacts. If there had previously been any supercoiling, this would be disrupted by the drug. An example used to demonstrate this theory is Actinomycin D. This small molecule binds between C-G and G-C base pairs. It inhibits replication of the genome and transcription of genes, by preventing the separation of the DNA strands. This drug was developed in Auckland and is a therapeutic treatment for leukemia.

In the early 1960s, Lerman described a number of physical studies on the interactions of DNA with planar aromatic cations. He concluded from these studies that planar aromatic molecules could bind to DNA. He proposed a mechanism where the aromatic molecules could slip in between the base pairs of DNA, a process that he termed as intercalation (Figure 20). This classical model has now been established for a large number of polycyclic aromatic compounds. As a result of rotation about torsional bonds
in the DNA backbone, the creation of an intercalation site causes separation of base-pairs and a lengthening of the double helix which can be detected by hydrodynamic methods, such as viscosity and sedimentation. Groove binding molecules do not significantly increase the viscosity of sonicated DNA or unwound DNA base pairs. In the classical model, the helix is lengthened by $3.4 \times 10^{-10}$ m maximum as a result of intercalation. The helix is unwound at the site of intercalation complex and the normal approximately 36° rotation of one base pair with respect to the next is decreased as a result of intercalation. The amount of unwinding depends upon the structure of intercalating molecules and probably on the DNA sequence in a manner that is not very well understood. The anthracycline drugs daunomycin and adriamycin unwind DNA by 11°, ethidium and propidium give unwinding angles of 26°, and proflavine and related acridines unwind DNA by 17° per bound molecule.

Crystallographic studies provide molecular detail concerning intercalators induced tilt and long-range compensating effects in the DNA duplex. In general, drugs such as proflavine and ethidium are stacked with their long axes parallel to the long axes of the adjacent base pairs in these crystal structures. The exocyclic amino groups of the intercalators point towards diester oxygens of the DNA phosphate groups at the intercalation site and provide additional electrostatic and hydrogen bonding stabilization of the complex. The base-pair centers are separated by 6.9 angstroms in the complex and stack well with the intercalated system.
Wang and coworkers obtained the first crystal structure with a nonintercalator and oligonucleotide of the antibiotic daunomycin and the oligomer d(GTG'TACG). Daunomycin binds to the duplex with its long axis almost perpendicular to the long axis of adjacent base-pairs at the intercalation site (Figure 21). The daunomycin amino-sugar, which is attached to ring A of the anthracycline ring system, lies in the minor groove of ring D, which bears a methoxy group, protrudes into the major groove. The daunomycin core, rings B and C, lies between base-pairs. The DNA has two daunomycin molecules at each CG unit present at the two ends. The AT base-pairs retain a general B-DNA-like geometry in the complex, but with some backbone distortions. As expected, the C/G intercalation site opens by 3.4 Å to create the intercalation space. The cationic amino-sugar substituent and ring largely fill the minor groove and displace water molecules and ions from it. Ring A has a hydroxyl group that can form a hydrogen bond with the N-3 of guanine and can accept a hydrogen bond from the amino group of the
same guanine. This results in favorable binding energy and orientational specificity for the intercalated drug.\textsuperscript{131}

The conformation of the duplex is also changed significantly relative to the B-DNA helical structure to accommodate the antibiotic. In addition to the increased separation of base-pairs at the intercalation site, the G-C base-pairs are also shifted laterally towards the major groove so that the helix axis changes position. At the intercalation site, the base-pairs maintain the usual 36\degree helical twist, but base-pairs at adjacent sites are unwound by 8\degree.\textsuperscript{133}

![Diagram of intercalation site](image)

Figure 22: Examples of classical intercalators: Daunomycin, R=H; Adriamycin, R=OH (left) and Ethidium bromide (right).

Most intercalators display either no binding preference or a slight G:C base-pair preference and this contrasts with the general A:T preference of outside binding compounds. It has been suggested that the general G:C preference of intercalators is due to the larger intrinsic dipole moment of G:C relative to A:T base-pairs and the resulting ability of G:C base-pairs to induce polarization into the ring system of intercalators.

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Nonetheless, intercalators with an A.T preference have been synthesized. This shows that overall binding specificity must depend not only on dipole interactions with polarizable intercalators but also on hydrogen-bonding interactions, and the size, hydration, and electrostatic potential of the grooves and other similar factors.

If the groove binding molecules and intercalators are compared, it is clear that groove-binders, at a class, display significantly greater binding selectivity than intercalators. Intercalation cavities created at A.T or G.C base-pairs are quite similar in their potential for interaction with planar aromatic ring systems. Electrostatic, van der Waals, hydrophobic, etc. contributions to binding are similar for the two sites. On the other hand, groove-binding molecules can contact more base-pairs as they lie along the groove in a DNA complex and this gives them an inherently greater recognition potential.

Simple intercalators with reduced steric constraints such as proflavine and ethidium bromide could potentially intercalate at every base pair site. Saturation of the double helix and intercalators would yield a binding stoichiometry of one base-pair per bound intercalator. While models of duplex DNA with reasonable backbone torsional angles can indeed be constructed with intercalators at all possible sites between base-pairs, solution studies indicate that even the simplest intercalators reach saturation at a maximum of one intercalator per two base-pairs. The neighbor exclusion principle states that intercalators can bind at alternative possible base-pair sites on DNA, giving a maximum of one intercalator between every second site. Initially, all spaces between base-pairs are potential binding sites for a non-specific intercalator. When an intercalator binds at one particular site, the exclusion principle states that it becomes impossible to

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bind another intercalator at either adjacent site. One suggestion for the molecular basis of neighbor exclusion is that intercalative binding induces conformational changes at adjacent sites in DNA and the new conformation makes it impossible to intercalate another mono-intercalator at the adjacent site.

An alternative theory was proposed by Friedman and Manning. According to them, for long pieces of DNA, as the intercalators bind to DNA, they neutralize some of its charge. This leads to a lower apparent binding constant since the favorable release of condensed ions is reduced, giving rise to a curvature in binding isotherms, similar to that predicted by neighbor exclusion. Since the local release of ions due to intercalation would be greater than ion release at a distance along the DNA molecule, the local release of counterions could also lead to reduced binding at neighboring sites. It is likely that both conformational as well as electrostatic forces contribute to the observed neighbor exclusion.

Non-classical Intercalators: Intercalators which have substituents on opposite sides of the ring system and must thread one of the substituents between the base-pairs at the intercalation site in the binding mechanism. If the substituent is bulky and/or polar or charged, this could be an unfavorable step, which might profoundly affect the kinetics of association and dissociation of the intercalator. Examples of such type of intercalators are naphthalene-bisimides cationic porphyrin.

The binding mechanism of simple intercalators such as proflavine involves two steps. First, the cationic intercalator interacts with DNA through an external electrostatic complex with the anionic backbone of the double helix. The intercalator then can diffuse into the anionic potential along the surface of the helix until it encounters the gaps.
between base-pairs which have separated as a result of thermal motion to create a cavity for intercalation. The molecule can then bind in an intercalation complex.\textsuperscript{130}

These types of molecules all have very high DNA binding constants, indicating, that once the side chain slides between the base pairs, the DNA molecule can assume a conformation that gives a very favorable free energy of complex formation. The side chains present a kinetic barrier to binding but have favorable interactions in the final complex after they have passed through the double helix. The kinetic barrier of binding depends on the size of the side chain, its orientation, and its polarity.\textsuperscript{141}

Porphyrins are particularly interesting compounds since they appear to intercalate in G-C rich regions of DNA, but bind in the minor groove at A-T rich regions. Their binding properties vary substantially with the cationic substituent and type of the metal bound centrally in the porphyrin. Oligonucleotide NMR studies by Marzilli, Wilson and coworkers have suggested that the intercalating porphyrins have very high selectivity for binding a CpG sequence.\textsuperscript{142} This is unusual since the porphyrins do not have the hydrogen-bonding capability observed for other molecules that show high selectivity for binding at specific DNA sequences.

ii. Groove-Binding Drugs

Medically, these are primarily molecular "blocking" devices. They prevent the binding of other proteins and enzymes to the area of DNA where they are bound to. In this case, the example used is Netropsin, which interacts specifically to B-DNA, but not A- or Z-DNA. It binds in the minor groove, displacing water molecules, and is a perfect
fit in A-T rich regions. It also prevents replication and transcription, by blocking the binding of polymerases to the DNA. It occupies approximately 6 base pairs along the helix.\textsuperscript{143}

The major and minor grooves differ significantly in electrostatic potential, hydrogen-bonding characteristics, steric effects and hydration. Most of the proteins and oligonucleotide molecules are specific towards the major groove while small groove binding molecules in general prefer the minor groove. Netropsin and distamycin are typical minor groove binders.\textsuperscript{144}

Typically, minor groove binding molecules have several simple aromatic rings such as pyrrole, furan, or benzene connected by bonds with torsional freedom. The minor groove is generally not as wide as A:T-rich relative to G:C-rich regions and may 'fit' aromatic molecules better at A:T than G:C sequences. A molecule, given the correct twist of its linked aromatic rings, can fit snugly into the minor groove and form van der Waals contacts with the helical chains of the groove.\textsuperscript{145} There is the possibility for hydrogen bonding between the bound molecule and A:T base-pairs at C-2 carbonyl oxygen of T and the N-3 nitrogen of A. Although similar groups are present at G:C base-pairs, the amino group of the G presents a steric block to hydrogen bond formation at N-3 of G and at the C-2 carbonyl of C. The hydrogen bond between the amino group of G and the carbonyl oxygen of C in G:C base-pairs lies in the minor groove and sterically inhibits the molecules from penetrating into this groove in G:C rich regions.\textsuperscript{146} Thus the aromatic rings of many groove binding molecules form close contacts with the H-2 of A in the minor groove of DNA. It has also been shown that the negative electrostatic

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potential is greater in the A:T minor groove than in the G:C rich regions of DNA and this provides additional important source for A:T specific minor groove binding of cations.  

A possibility with groove binding molecules that does not exist with intercalators is that they can be extended to fit over many base-pairs along the groove and have high sequence specific recognition of nucleic acids. For example, an oligopyrimidine can be designed which forms a triple helix with a specific homopurine-homopyrimidine duplex sequence. By linking DNA cleavage reagents to the oligopyrimidine, a highly specific 'nuclease' can be created.  

iii. Electrostatic interactions

Condensation type interactions: These interactions usually involve the binding along the exterior of the helix through interactions that are generally non-specific. Manning et al. have shown that B-DNA is considered as a line of equally spaced charges, with one anionic charge per 1.7 angstrom. If the line charge spacing becomes less than approximately 7 angstrom, the molecule becomes unstable and associates with cations such as Na⁺, K⁺ from solution to achieve stability. The association of ions with the polyelectrolyte is called counterion condensation and causes an unfavorable entropy term in the overall polymer conformational free energy summation. This unfavorable term is overcome by various other favorable interactions in the DNA double helix. Counterions 'condense' until the charge density is reduced to the stable level of approximately one charge per 7 angstroms. Additional counterions are associated with the remaining charges on the polyanion through Debye-Huckel type interactions. For a specific
nucleic acid conformation, the charge density and thus the amount of condensed counterion is constant. The initial association of counterions is referred to as condensation, since the ions are associated with the general charge density of the polyelectrolyte and are not bound at specific sites. The ions retain their inner sphere water of hydration and move rapidly along the sugar-phosphate backbone of DNA.\textsuperscript{130} Secondary hydration layers of both the polyelectrolyte and counterion are affected by this interaction.

**Outside Stacking:** Planar aromatic molecules can stack on each other to form dimers and higher aggregates. When compounds are charged, such as protoporphyrin and porphyrins, they repel each other electrostatically.\textsuperscript{131} If, however, cations stack along the anionic DNA sugar-phosphate chain, the charge repulsion is decreased and this type of binding leads to nonspecific outside stacking of planar cations along the double helix. A large fraction of condensed counterions are released when such stacking takes place. It is therefore highly dependent on salt concentration and is generally quite weak at salt concentration of 0.1 M and above. Because this binding mode is a type of extended self-association, it can be highly cooperative and will generally be more favorable at high ratios of aromatic cation to DNA phosphate.\textsuperscript{132}

**VII. Porphyrins and DNA**

The first evidence that porphyrins are capable of intercalating into DNA came in 1970 by Fiel et al. reporting that the tetracationic meso-substituted porphyrin, 5,10,15,
20-tetraakis(4-N-methylpyridyl)-porphine (TMPyP4) can intercalate into calf thymus DNA (Figure 22). 133

This result was received with some surprise and even skepticism given the structural features of this molecule: X-ray crystal structures show the plane of N-methylpyridyl group to be at a 66-72° angle with respect to the main porphyrine core and NMR studies indicate that the rotational barrier for these N-methylpyridyl groups to obtain coplanarity with the porphyrine core is relatively large. 134

Thus, considerable steric constraints appear to exist for passage of these molecules between the base-pairs of DNA helix to form an intercalated complex. Since the original report indicating porphyrin intercalation appeared, a number of studies utilizing a wide range of techniques have been conducted and a detailed picture of the mode(s) and mechanism(s) of this binding process has emerged. 135

Pasernack and his group have studied in great detail the interactions of TMPyP4 and their metal derivatives with synthetic DNA. Their work is primarily responsible for the use of porphyrins and their metal counterparts as probes for studying drug-DNA interactions. On the basis of visible absorption spectroscopy, circular dichroism spectroscopy and kinetic studies, they suggested that TMPyP4 and its metal derivatives without the axial ligands[Au(III), Cu(II), Ni(II)] are capable of intercalation when interacting with poly(dG-dC). 136 It was reported that the intercalation process was accompanied by large bathochromic shift and substantial hypochromicity of the Soret band, a negative induced CD band in the Soret region of the porphyrin chromophore and binding kinetics that can be measured by temperature jump relaxation techniques. These same non-axially liganded porphyrins were shown to bind externally to poly(dA-dT).

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The externally bound complexes displayed moderate bathochromic shifts and hypochromicity (in some cases a hyperchromicity) and a positive induced CD band in the Soret region of the porphyrin chromophore.\textsuperscript{157}

Mn(III) porphyrins were shown not to intercalate into either poly(dG-dC)\textsubscript{2} or poly(dA-dT)\textsubscript{2} but bind externally to both polymers. It was suggested that the inability of these metalloporphyrins to intercalate was probably due to the additional steric requirements imposed by their axial ligands. Carlia, and coworkers considered binding patterns for the positional isomers of TMPyP4 at conditions similar to those of the above studies, importantly at low drug load (i.e. $\left[\text{porphyrin}\right]/\left[\text{DNA}\right]$ is small).\textsuperscript{158}

Based upon unwinding studies of covalently closed supercoiled DNA (ccs DNA), the 3-N methyl isomer metalloporphyrins with axial ligands [Zn(II), Co(III), Fe(III)], and TMPyP3 were shown to be intercalators. However, TMPyP2, in which the N-methyl group is in the ortho position (Figure 22), and H\textsubscript{2}TAP (meso-tetrais(N\textsubscript{2}N\textsubscript{2}N\textsubscript{3}-trimethyl anilinium)porphine), another cationic porphyrin, do not unwind ccs DNA which is typical for non-intercalators.\textsuperscript{159} As would be predicted for external with Poly(dG-dC)\textsubscript{2} (at low $c_0$) both display positive induced CD bands. All three species (TMPyP3, TMPyP2, H\textsubscript{2}TAP) exhibit positive induced CD bands with poly (dA-dT)\textsubscript{2} as does TMPyP4, again indicative of external groove binding. Therefore, the binding mode preferences of cationic meso substituted porphyrins can be modulated not only by changes in the coordinated metal but also by alterations in the porphyrin periphery.\textsuperscript{160}

It has also been shown that, for the interaction of TMPyP4 with calf thymus DNA, at low values of drug load ($c_0 = [\text{drug}]/[\text{DNA}] = 0.1$), the mode of binding is intercalative at low ionic strength and external at high ionic strength.\textsuperscript{161} At higher drug
loads (r<sub>n</sub>&#x22650.2), the binding of TMPyP<sub>4</sub>, TMPyP<sub>2</sub>, and H<sub>2</sub>TAP to DNA becomes more complex and the resultant CD spectra become more complicated. These effects are due to the conformational changes of the polymers, stacking of externally bound molecules on the surface of the DNA, aggregation of the polymers, and unavailability of more sites as a result of intercalative sites leading to outside binding.<sup>162</sup>

Marzilli and coworkers have studied the binding of TMPyP<sub>4</sub> with poly(dG-dC) and poly (dA-dT) by equilibrium dialysis and stopped-flow dissociation kinetics as a function of the concentration of Na<sup>+</sup>. Rates of dissociation from poly (dA-dT) were similar for TMPyP<sub>4</sub>, ZnTMPyP<sub>4</sub> and NiTMPyP<sub>4</sub>, which have similar mode of binding. The dissociation rates of NiTMPyP<sub>4</sub> dissociation rates from poly(dG-dC) were measurable while the dissociation of ZnTMPyP<sub>4</sub> dissociation was too fast to measure (outside binder). Increasing the [Na<sup>+</sup>] from 0.115 M to 0.515 at 20°C increased the fast and slow dissociation rate constants by a factor of 204 for TMPyP<sub>4</sub> dissociation from poly (dG-dC) indicating an intercalative mode of binding.<sup>163</sup> DNase was used to study the footprinting of different porphyrin-DNA complexes.<sup>164</sup> DNase digestion produces a larger degree of cutting at the GC sites and small edges of cutting at the AT sites. The lesser degree of cleavage of AT sites is indicative of externally bound porphyrin, which protect the AT sites from cleavage.
Figure 23: Structures of different porphyrins and their metal derivatives.
Conversely, intercalated porphyrins in the GC sites afford no protection for the DNA against DNase that is seen as larger amount of cleavage products in the footprinting assay.\textsuperscript{165}

As found with other intercalating drugs, porphyrins and metalloporphyrins are capable of converting Z-form DNA to the right-handed B conformation. Kinetic studies imply that these porphyrin species intercalate into Z-form prior to B formation although some uncertainty exists as to the nature of drug-Z-DNA complex. It has been suggested that conversion of DNA between Z and B forms could be one mechanism for control of genetic process.\textsuperscript{166} If this is the case then these intercalating porphyrins could interfere with such control mechanisms by binding to Z-regions of DNA and converting them to the B-form or by preventing conversion of B-DNA to the Z conformation in vivo.

Aggregation studies on the effects of meso-substituted porphyrins by light scattering techniques have been done.\textsuperscript{167} Addition of NaCl to aqueous solutions of t-H_{2}Pagg or t-CuH_{2}Pagg [\textit{trans-cis}(p-methylpyridinium-4-yl)]diphenylporphyrine and its Cu(II) derivative], initially at an ionic strength of zero, leads to a large bathochromic shift and hypochromism of the Soret band indicating extensive aggregation of these porphyrins. Extensive scattering was observed for t-H_{2}Pagg with the scattering profile showing a wavelength dependence characteristic of the spectrum of the scattering species with an absorption maximum at 455 nm.\textsuperscript{168} The extent of the scattering is dependent on the ionic strength in a manner to be expected for a self-aggregation process involving charged species. The most dramatic effect arising from the complexation to nucleic acids by the t-H_{2}Pagg and t-CuH_{2}Pagg porphyrin derivatives under 0.1 M NaCl is the generation of circular dichroism signals 1-2 orders of magnitude larger than those observed under low

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salt conditions. Paternack suggested that unaggregated DNA could serve as a template on which t-H3Pagg and t-CuH3Pagg form long-range chiral assemblies.218

CuTMPyP is the square planar complex of TMPyP4 and has no axial ligands. The porphyrin intercalates between the C and the G of 5' CGA 3' and excludes the C of the 5'CGA 3'. Only four normal Watson-Crick base pairs are observed in the DNA-porphyrin complex.164 In addition, the DNA forms a disorted right-handed helix. Electrostatic interactions between positively charged nitrogen atoms of the porphyrin and the oxygen atoms of the phosphate backbone of the DNA seemed to extensively stabilize the DNA-porphyrin complex. These favorable interactions offset the unfavorable steric clashes between the pyridine rings of the porphyrin and DNA backbone. The pyridine backbone clashes along the DNA along its axis, which reduces the stacking interactions in the interior of the DNA-porphyrin complex that was observed as decreased energetic cost of local melting.176

VIII. Porphyrins and DNA quadruplexes

The development of agents for the structure specific recognition of DNA quadruplexes has largely stemmed from experience with intercalators for duplex or triplex DNA. Most of these compounds are based upon planar-extended aromatic ligands where DNA binding is anticipated via molecular x-overlap with the G-tetrad planes. Qualitative support for this approach was provided by a report that the dye etidium bromide binds to intermolecular quadruplexes by a mode consistent with intercalation.177
The cationic tetra-(N-taethy-4-pyridyl)porphyrine has recently been shown to bind to quadruplex-form DNA and inhibit telomerase activity. A wide range of related porphyrin derivatives have since been examined, providing a basis for early structure-activity relationships. However, while it is established that these agents bind to quadruplex DNA and that this binding must be implicated in the telomerase inhibition, the mode(s) of binding are by no means unambiguous. The stoichiometries, affinities and sites for binding remain controversial, with alternative intercalated, end-pasted or sandwich-type interaction modes. While the mechanism of binding is still unclear, it is already known that the binding behavior is also influenced by the solution conditions and the DNA complexation. In addition, caution should be taken in comparing binding properties from alternative and dissimilar assay methods.

Studies of the interaction of TMPyP4 with the parallel quadruplex formed by TGG, and with the duplex formed by d(CCGCATATCGCG) have been conducted by Sheardy and coworkers. Results from visible absorption spectroscopy, circular dichroism, and fluorescence energy transfer experiments showed that TMPyP4 binds to both the duplex and quadruplex with high affinity. While this particular porphyrin was shown to bind to duplex DNA via intercalation, the mode of binding for quadruplex DNA was suggested to be both intercalative as well as end passing forming a "sandwich" type complex involving one porphyrin stacked between the 5' termini G-tetrads of two quadruplexes.

The interaction between porphyrin with three quadruplexes has been studied using calorimetry, molecular modeling and spectrophotometry. These studies showed that the binding affinity is rather weak and that the binding stoichiometry corresponds exactly to the number of intervals between stacked G-tetrads in each DNA structure (e.g., the
human d[AGGG(TTAGGG)]₆ quadruplex with three G-tetrahedron planes will bind two porphyrins. According to the thermodynamic parameters obtained, they suggested the binding mode to be intercalative rather than end-pasted or sandwich type binding.¹⁷³

Bolton and his coworkers studied the interaction of anionic N-methyl mesoporphyrin NMM, and some other porphyrins with different DNA quadruplexes including aptamer. Their fluorescence energy transfer experiments suggest that the porphyrin NMM favors the binding to quadruplex DNA rather than duplex form DNA and so could offer a route to the specific detection of quadruplex DNA under biologically important conditions. In other studies done by NMR, they have shown that at high DNA concentrations, certain porphyrins catalyze the conversion of both chair and basket type structures into parallel strand quadruplex DNA.⁹⁶

It has also been shown that the fluorescence of selected porphyrins can be used to discriminate between duplex and quadruplex DNA as well as between different secondary structures of DNA quadruplexes.⁶⁷

In another study done by Hurley and coworkers, interactions of TMPyP4 and TMPyP2 with an antiparallel quadruplex DNA were studied. Both of these cationic porphyrins posses similar structures but have been shown to have strikingly different potencies for telomerase inhibition. A single stranded DNA oligonucleotide (G₄A) containing four human telomere repeats of GGGTTA was designated to form an intramolecular quadruplex structure. Thermal melting profiles show that both TMPyP4 and TMPyP2 stabilize quadruplex DNA to about the same extent. The photocleavage assay shows that TMPyP4 binds by stacking externally to the guanine tetrad at the GT step, while TMPyP2 binds predominantly to the same G₄A DNA structure via external
binding to the YTA loop and this difference in binding locations accounts for differential effects on telomerase inhibition by both porphyrins.178
CHAPTER III
THEORY, METHODS AND MATERIALS

i: Theory

The recognition of DNA at a molecular level is one the most important discoveries of science. The ability to study the interaction of small molecules with DNA and being able to understand how these ligands bind to DNA is the key to designing new novel molecules for the treatment of diseases. Research in the past 20 years has been focused on the type of binding between DNA and small molecules. Also, the thrust has been focused on the main types of noncovalent small DNA interactions as it pertains to intercalation, groove binding and simple electrostatic interactions.¹⁷⁹

The discovery that simple tandemly-repeated G-rich telomeric DNA is preserved in most eukaryotes and that these repeats can fold into four-stranded quadruplex structures, spurred a growth in research in this area which has created a greater understanding and has stimulated new discoveries during the past 20 years. Quadruplexes are believed to play a vital cellular role, despite their lack of detection in vivo, and the preservation of telomeric DNA sequences may be related to their inherent quadruplex formation.¹⁸⁰ This hypothesis is supported by recent findings that many telomeric DNA binding proteins bind to quadruplexes and or promote their formation and may also regulate telomerase activity. Moreover, it is now established that the RNA template region of telomerase requires a linear unfolded telomeric DNA primer to effect telomere
elongation. Consequently, the presence of a telomeric quadruplex structure leads to the inhibition of telomerase activity. Such an identification of DNA quadruplexes as a target for the development of telomerase inhibitors has aroused the recent search for new ligands that can selectively stabilize DNA quadruplexes and hence eventually could be used to inhibit the activity of telomerase. The key step towards such formulations, however, is to have a better understanding of the structure and conformation of these DNA quadruplex systems, which are extremely sensitive to the sequence of DNA as well as the environmental conditions. In addition, stoichiometries, binding affinities, and the mode of binding of ligands to these quadruplexes are the questions to be addressed in order to optimize the efficacy of such agents and to prevent their unwarranted depletion by competitor DNA target sites.

The purpose of this study was to synthesize porphyrines and to study the binding of different porphyrines to DNA quadruplexes, DNA duplexes and DNA single strands. The present study used atomic force microscopy, competitive dialysis and uv/vis spectroscopy to investigate if specific porphyrines would interact with certain DNA quadruplexes, DNA Duplexes and DNA single stranded oligonucleotides and to see if the porphyrines have any utility as potential AIDS and cancer inhibitors. The essence of his work is to expand the utility of porphyrines as potential cancer and AIDS pharmaceutical agents and to expand the current database with respect to DNA quadruplexes and to understand how porphyrines can stabilize DNA.
DNA Synthesis

Each self-complementary DNA strand was synthesized by the automated phosphoramidite method of the Applied Biosystems 380B DNA synthesizer (Foster City, CA). The synthesis is done with the growing nucleoside chain attached to a solid Controlled Pore Glass (CPG) support so that excess reagents that are in a liquid phase can be removed by filtration. At the starting material, the solid support is derivatized with the nucleoside that will be the 3'-hydroxyl end of the product. The nucleoside is bound to the silica solid support through a long spacer arm attached at the 3'-hydroxyl via an ester bond, which is base labile and allows for removal of the DNA from the support. All free silanol groups are capped to prevent side reactions. The 5'-hydroxyl is blocked with a dimethoxytrityl group. Thus, automated DNA synthesis builds the oligonucleotide chain 3' to 5'. The use of the solid support is essential to the automation of DNA synthesis because it eliminates the need for purification steps between base additions.

The first step of the synthesis cycle is treatment of the derivatized solid support with acid to remove the trityl group and free the 5'-hydroxyl for the addition reaction. The next step, activation, creates a highly reactive nucleoside derivative that reacts with the hydroxyl group. The intermediate is created by simultaneously adding the phosphoramidite derivative of the next nucleoside and a weak acid, tetrazole, to the reaction chamber. The tetrazole protonates the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack. Since this intermediate is so reactive, the addition reaction is complete in less than 30 seconds at room temperature. The added phosphoramidite is blocked at the 5'-hydroxyl end with the dimethoxytrityl group. The
exocyclic amines on adenosine (A) and cytosine (C) are protected by a benzoyl group and the exocyclic amine on guanosine (G) by an isobutyryl group. Thymidine is already unreactive and does not need a protecting group.

The next step, capping, terminates any chains that did not undergo addition. Since the unreacted chains have a free 5'-hydroxyl, they can be terminated or capped by acetylation. This reaction is accomplished with acetic anhydride and dimethylaminopyridine. Since the chains that reacted with the phosphoramidite in the previous step are still blocked with the dimethoxytrityl group, they are not affected by this step. Although capping is not required for DNA synthesis, it minimizes the lengths of the impurities and thus facilitates the purification by Reverse Phase-HPLC.

The internucleotide linkage is then converted from the phosphite to the more stable phosphate. Iodine is used as the oxidizing agent and water as the oxygen donor. This reaction is completed in less than 30 seconds. After the oxidation step, the dimethoxytrityl group is removed by acid hydrolysis, and the cycle is repeated until chain elongation is complete. At this point, the oligonucleotide is still bound to the support and has protecting groups on the phosphates and the exocyclic amines of the bases A, G, and C. The chain is then cleaved from the support by a one-hour treatment with concentrated ammonium hydroxide. After the solution containing the DNA is removed from the instrument, the protecting groups on the exocyclic amines of the bases are cleaved by an 18-hour treatment with concentrated ammonium hydroxide at 55°C.
Porphyrazines and General methods of preparation

The synthesis of porphyrazine derivatives are of great interest due to their applications in liquid crystals, chemical sensors, and nonlinear optics. The emerging field of photodynamic cancer therapy has also prompted interest in porphyrazine derivatives as potential target molecules for treating cancer.

Cationic porphyrins and porphyrazines, metallated and nonmetallated, have also attracted much attention as photosensitizers for photodynamic cancer therapy based on their DNA binding properties. The porphyrin ring system has been one of the most studied macrocyclic ring systems. Research in this naturally occurring tetrapyrrolic macrocycle is based and derives in part from its biological functions as well as its ability to be an excellent metal-complexing ligand. This chemical has opened the study of a range of porphyrin analogues. The thrust in research devoted to these latter systems is based on their resemblance to porphyrins and the vision that they will show a rich coordination chemistry that parallels that of the porphyrins. Also, the electronic structure of various larger porphyrin systems, in particular their resemblance and differences to porphyrins, have made them an area of intense study.

The uv-vis study of conjugated expanded porphyrins display absorbance bands that are considerably red-shifted relative to those of porphyrin, and this unique chemical property makes them perfect candidates as photodynamic therapeutic agents.

In recent years, unsubstituted and symmetrically substituted phthalocyanines, tetrabenzoporphyrines, napththalocyanines have been researched as second generation photosensitizers due to increased p-conjugation, this has led to the theory
that certain expanded porphyrins could find use as therapeutics for photodynamic therapy. The properties, including ease of reduction, has resulted in the testing of one particular expanded porphyrin (xycrin, motexafin gadolinium) as an adjuvant for X-ray radiation therapy. Another area of interest in the porphyrin field has been focused around the discovery that expanded porphyrins can act as anion receptors. These findings have made these systems a potential area of interest for use in a variety of applications, including anion sensing and transport for drug delivery, as well as chromatography-based purification of anions.

Metallated or unmetallated phthalocyanines, are referred to as phthalocyanine (pc), are usually prepared from phthalonitrile or from 1,3-diminoisoindoline Twigg and coworkers heated phthalonitrile in methylphosphatene or other high boiling solvents in the presence of catalytic amounts of cyclohexylamine. Other amines such as lanthanum chloride triethylammoniate has been used. Heating phthalonitrile in dioxane at 200 °C under hydrogen pressure gives moderate yields. Tetraphenyl-pc is also prepared by refluxing 4-phenylphthalonitrile, with a catalytic amount of ammonium molybdate, and isoamyl alcohol containing sodium for 5 hours. After filtration the product is stirred with ethanol for 10 hours, filtered and dried. Naphthalocyanine is made in the same manner and then is converted to many different metal phthalocyanines. Reacting phthalocyanines with complex alcohodates results in yields ranging from 4% to 81% when using lithium methoxide in the presence of tetrafluoroethane as the solvent at 180 °C.

Pc's can also be prepared by heating 1,3-diminoisoindoline in an organic heteropolar solvent like isoamyl alcohol and dimethylformamide in the presence of
reducing agents such as sodium sulfide. A mixture of 1,3-diminoisoindoline nitrate with an 80% solution of urea in water, stirred in the presence of sodium hydrosulfide, results in phthalocyanines. Another method used in the preparation of Pc’s requires, reacting diminoisoindoline with dimethylformamide and dichloroisindole in benzene and refluxing for 9 hours, filtering, and treating the residue with aqueous sodium hyposulfite. Pc’s may be purified by boiling with sodium chloride and HCl solutions, followed by heating in a stream of inert gas, followed by sublimation resulting in the improvement of electrophysical characteristics. Pc’s can also be purified by refluxing in a strong base in alcoholic solution.

UV/Vis Spectroscopy

Electromagnetic radiation is composed of photons moving in a wave that oscillates along the path of motion. The wavelength of light is defined through the equation \( \lambda = c/v \) where \( \lambda \) is the wavelength, \( c \) is the speed of light and \( v \) is the frequency. Photons of different wavelengths have different energies that are given by: \( E = hc/\lambda = hv \) where \( h \) is the Max Planck constant. The shorter the wavelength, the greater the energy of the photon. Electromagnetic radiation can be divided into various regions according to wavelength: the ultraviolet region has wavelengths of 200-400 nm, the visible region has wavelengths of 400-700 nm.

Molecules have kinetic energy and energy associated with the bonding of electrons. The absorption of light affects the energy of bonding electrons, but it does not affect the kinetic energy. Absorption of electromagnetic radiation involves a change in one or more bonding energies. A molecule whose electronic, vibrational
and rotational energies are all at their lowest values is said to be in the ground state. When a molecule is irradiated, the photons may be absorbed and the molecule raised to a higher energy level. Whether any electronic transition can occur in the visible or ultraviolet region depends on the nature of bonding electrons in the ground state. In general, the absorption of ultraviolet and visible light takes place with molecules with chromophoric groups such as purines and pyrimidines. Any molecule containing one or more of such groups will have an absorption band somewhere in the visible or ultraviolet regions.

For a single cuvette path length, the transmitted intensity decreases exponentially with increasing concentration of an absorbing solute. The absorbance is defined as $A = \log(I/I_0) = \epsilon c l$, where $A$ is the absorbance or optical density, $I_0$ is the intensity of incident light, $I$ is the transmitted intensity, and $c$ is the molar absorptivity (or molar extinction coefficient) with units M$^{-1}$ cm$^{-1}$, and the concentration $c$, is in molarity (M). The Beer-Lambert law shows that absorbance is linearly related to concentration (or path length).

Since absorption strongly depends on wavelength for nearly all compounds, we must specify the wavelength at which measurement is made. This is done using a subscript $\lambda$, indicating the particular wavelength, as $A_\lambda$ or $c_\lambda$. For a single substance at a specified wavelength, $c_\lambda$ is a constant, characteristic of the absorbing sample, and is independent of both $c$ and $l$. The wavelength dependence of $c_\lambda$ or $A_\lambda$ is known as the absorption spectrum of the compound. Deviation from the Beer-Lambert law can occur for a variety of reasons such as inhomogeneous samples, light scattering by the
sample, dimerization or other aggregation at high concentrations or changes in equilibria involving dissociable absorbing solutes. 203

A wavelength at which two or more components have the same extinction coefficient is known as an isosbestic wavelength, \( \varepsilon_3, M = \varepsilon_3, N = \varepsilon_3, m \). At this wavelength, the absorbance can be used to determine the total concentration of the two components. Measurements at an isosbestic wavelength plus one other wavelength where the extinction coefficients differ for the two components provide a particularly simple solution to their Beer-Lambert law equations:

\[
\begin{align*}
[M] & = \frac{\varepsilon_3, A_m - \varepsilon_3, A_n}{\varepsilon_3, A_k - \varepsilon_3, A_m} \\
[N] & = \frac{\varepsilon_3, A_m - \varepsilon_3, A_n}{\varepsilon_3, A_k - \varepsilon_3, A_m}
\end{align*}
\]  

(1)

Two common uses of isosbectics are the study of equilibria involving absorbing compounds and investigations of reactions involving absorbing reactants and products. The presence of isosbectics is used as evidence that there are no intermediate species of significant concentration between the reactants and products. 204

Binding Constants:

The three simplest complex stoichiometries are \( SL \), \( SL_2 \) and \( SL_3 \). If it is assumed that every complex is formed in a bimolecular process, then the three complexes are related by the following equilibria:

\[
\begin{align*}
S + L & \rightleftharpoons SL \\
SL + L & \rightleftharpoons SL_2 \\
S + SL & \rightleftharpoons SL_3
\end{align*}
\]
DNA Synthesis

Each self-complementary DNA strand was synthesized by the automated phosphoramidite method of the Applied Biosystems 380B DNA synthesizer (Foster City, CA). The synthesis is done with the growing nucleotide chain attached to a solid Controlled Pore Glass (CPG) support so that excess reagents that are in a liquid phase can be removed by filtration. At the starting material, the solid support is derivatized with the nucleoside that will be the 3′-hydroxyl end of the product. The nucleoside is bound to the silica solid support through a long spacer arm attached at the 3′-hydroxyl via an ester bond, which is base labile and allows for removal of the DNA from the support. All free silanol groups are capped to prevent side reactions. The 5′-hydroxyl is blocked with a dimethoxytrityl group. Thus, automated DNA synthesis builds the oligonucleotide chain 3′ to 5′. The use of the solid support is essential to the automation of DNA synthesis because it eliminates the need for purification steps between base additions.

The first step of the synthesis cycle is treatment of the derivatized solid support with acid to remove the trityl group and free the 5′-hydroxyl for the addition reaction. The next step, activation, creates a highly reactive nucleoside derivative that reacts with the hydroxyl group. The intermediate is created by simultaneously adding the phosphoramidite derivative of the next nucleoside and a weak acid, tetrazole, to the reaction chamber. The tetrazole protonates the nitrogens of the phosphoramidite, making it susceptible to nucleophilic attack. Since this intermediate is so reactive, the addition reaction is complete in less than 30 seconds at room temperature. The added phosphoramidite is blocked at the 5′-hydroxyl end with the dimethoxytrityl group.
exocyclic amines on adenosine (A) and cytosine (C) are protected by a benzoyl group and the exocyclic amine on guanosine (G) by an isobutyryl group. Thymidine is already unreactive and does not need a protecting group.

The next step, capping, terminates any chains that did not undergo addition. Since the unreacted chains have a free 5'-hydroxyl, they can be terminated or capped by acetylation. This reaction is accomplished with acetic anhydride and dimethylamino pyridine. Since the chains that reacted with the phosphoramidite in the previous step are still blocked with the dimethoxytrityl group, they are not affected by this step. Although capping is not required for DNA synthesis, it minimizes the lengths of the impurities and thus facilitates the purification by Reverse Phase-HPLC.

The internucleotide linkage is then converted from the phoshphite to the more stable phosphate. Iodine is used as the oxidizing agent and water as the oxygen donor. This reaction is completed in less than 36 seconds. After the oxidation step, the dimethoxytrityl group is removed by acid hydrolysis, and the cycle is repeated until chain elongation is complete. At this point, the oligonucleotide is still bound to the support and has protecting groups on the phosphates and the exocyclic amines of the bases A, G, and C. The chain is then cleaved from the support by a one-hour treatment with concentrated ammonium hydroxide. After the solution containing the DNA is removed from the instrument, the protecting groups on the exocyclic amines of the bases are cleaved by an 18-hour treatment with concentrated ammonium hydroxide at 55°C.
Porphyrazines and General methods of preparation

The synthesis of porphyrazine derivatives are of great interest due to their applications in liquid crystals, chemical sensors, and nonlinear optics. The emerging field of photodynamic cancer therapy has also prompted interest in porphyrazine derivatives as potential target molecules for treating cancer.

Cationic porphyrins and porphyrazines, metallated and nonmetallated, have also attracted much attention as photosensitizers for photodynamic cancer therapy based on their DNA binding properties. The porphyrin ring system has been one of the most studied macrocyclic ring systems. Research in this naturally occurring tetrpyrrolic macrocycle is based and derives in part from its biological functions as well as its ability to be an excellent metal-complexing ligand. This chemical has opened the study of a range of porphyrin analogues. The thrust in research devoted to these latter systems is based on their resemblance to porphyrins and the vision that they will show a rich coordination chemistry that parallels that of the porphyrins. Also, the electronic structure of various larger porphyrin systems, in particular their resemblance and differences to porphyrins, have made them an area of intense study.

The UV-vis study of conjugated expanded porphyrins display absorbance bands that are considerably red-shifted relative to those of porphyrin, and this unique chemical property makes them perfect candidates as photodynamic therapeutic agents.

In recent years, unsubstituted and symmetrically substituted phthalocyanines, tetrabenzoporphyrizes, napththocyanines have been researched as second generation photosensitizes due to increased p-conjugation, this has led to the theory
that certain expanded porphyrins could find use as therapeutics for photodynamic therapy.\textsuperscript{189} The properties, including ease of reduction, has resulted in the testing of one particular expanded porphyrin (s-cytrin, motexafin gadolinium) as an adjuvant for X-ray radiation therapy. Another area of interest in the porphyrin field has been focused around the discovery that expanded porphyrins can act as anion receptors.\textsuperscript{189} These findings have made these systems a potential area of interest for use in a variety of applications, including anion sensing and transport for drug delivery, as well as chromatography-based purification of anions.\textsuperscript{190}

Metallated or unmetallated phthalocyanines, are referred to as phthalocyanine (pc), are usually prepared from phthalonitrile or from 1,3-diminoisoindoline. Twigg and coworkers heated phthalonitrile in methylphthalalene or other high boiling solvents in the presence of catalytic amounts of cyclohexylamine.\textsuperscript{191} Other amines such as lanthanum chloride triethyloximate has been used. Heating phthalonitrile in dioxane at 200 °C under hydrogen pressure gives moderate yields. Tetraphenyl-pc is also prepared by refluxing 4-phenylphthalonitrile, with a catalytic amount of ammonium molybdate and isomyl alcohol containing sodium for 5 hours. After filtration the product is stirred with ethanol for 10 hours, filtered and dried.\textsuperscript{192} Naphthalocyanine is made in the same manner and then is converted to many different metal phthalocyanines. Reacting phthalocyanines with complex anilolates results in yields ranging from 4% to 81% when using lithium methoxide in the presence of tetralin as the solvent at 180 °C.\textsuperscript{193}

Pc's can also be prepared by heating 1,3-diminoisoindoline in an organic heteropolar solvent like isomyl alcohol and dimethylformamide in the presence of
reducing agents such as sodium sulfide. A mixture of 1,3-diminoisoindoline nitrate with an 80% solution of urea in water, stirred in the presence of sodium hydroxide, results in phthalocyanines. Another method used in the preparation of pC's requires, reacting diminoisoindoline with dimethylformamide and dichloroisindole in benzene and refluxing for 9 hours, filtering, and treating the residue with aqueous sodium hyposulfite. pC's may be purified by boiling with sodium chloride and HCl solutions, followed by heating in a stream of inert gas, followed by sublimation resulting in the improvement of electrophysical characteristics. pC's can also be purified by refluxing in a strong base in alcoholic solution.

UV/VIS Spectroscopy

Electromagnetic radiation is composed of photons moving in a wave that oscillates along the path of motion. The wavelength of light is defined through the equation $\lambda = c/\nu$ where $\lambda$ is the wavelength, $c$ is the speed of light and $\nu$ is the frequency. Photons of different wavelengths have different energies that are given by:

$$E = h\nu = h/\lambda$$

where $h$ is the Max Planck constant. The shorter the wavelength, the greater the energy of the photon. Electromagnetic radiation can be divided into various regions according to wavelength: the ultraviolet region has wavelengths of 200-400 nm, the visible region has wavelengths of 400-700 nm.

Molecules have kinetic energy and energy associated with the bonding of electrons. The absorption of light affects the energy of bonding electrons, but it does not affect the kinetic energy. Absorption of electromagnetic radiation involves a change in one or more bonding energies. A molecule whose electronic, vibrational
and rotational energies are all at their lowest values is said to be in the ground state. When a molecule is irradiated, the photons may be absorbed and the molecule raised to a higher energy level. Whether any electronic transition can occur in the visible or ultra violet region depends on the nature of bonding electrons in the ground state. In general, the absorption of ultraviolet and visible light takes place with molecules with chromophoric groups such as purines and pyrimidines. Any molecule containing one or more of such groups will have an absorption band somewhere in the visible or ultraviolet region.

For a single cuvette path length, the transmitted intensity decreases exponentially with increasing concentration of an absorbing solute. The absorbance is defined as $A = \log(I_0/I) = -\varepsilon c d$ where $A$ is the absorbance or optical density, $I_0$ is the intensity of incident light, $I$ is the transmitted intensity, and $\varepsilon$ is the molar absorptivity (or molar extinction coefficient) with units M$^{-1}$ cm$^{-1}$, and the concentration $c$, is in molarity (M). The Beer-Lambert law shows that absorbance is linearly related to concentration (or path length).

Since absorption strongly depends on wavelength for nearly all compounds, we must specify the wavelength at which measurement is made. This is done using a subscript $\lambda$, indicating the particular wavelength, as $A_\lambda$ or $\varepsilon_\lambda$. For a single substance at a specified wavelength, $\varepsilon_\lambda$ is a constant, characteristic of the absorbing sample, and is independent of both $c$ and $I$. The wavelength dependence of $\varepsilon_\lambda$ or $A_\lambda$ is known as the absorption spectrum of the compound. Deviation from the Beer-Lambert law can occur for a variety of reasons such as inhomogeneous samples, light scattering by the
sample, dimerization or other aggregation at high concentrations or changes in equilibria involving dissociable absorbing solutes.  

A wavelength at which two or more components have the same extinction coefficient is known as an isosbestic wavelength. \([\epsilon_M = \epsilon_N = \epsilon_{\infty}]\). At this wavelength, the absorbance can be used to determine the total concentration of the two components. Measurements at an isosbestic wavelength plus one other wavelength where the extinction coefficients differ for the two components provide a particularly simple solution to their Beer-Lambert law equations:

\[
\frac{[M]}{[N]} = \frac{e_M^M A_m - e_M^N A_N}{e_N^M A_m - e_N^N A_N} \tag{1}
\]

Two common uses of isosbetics are the study of equilibria involving absorbing compounds and investigations of reactions involving absorbing reactants and products. The presence of isosbetics is used as evidence that there are no intermediate species of significant concentration between the reactants and products.

**Binding Constants:**

The three simplest complex stoichiometries are \(SL, SL_2\) and \(S_2L\). If it is assumed that every complex is formed in a bimolecular process, then the three complexes are related by the following equilibria:

\[
\begin{align*}
S + L & \rightleftharpoons SL \\
SL + L & \rightleftharpoons SL_2 \\
S + SL & \rightleftharpoons S_2L
\end{align*}
\]
We define the stepwise binding constants as:

\[
K_{11} = \frac{[SL_1][S][L_1]}{[S][L][SL_1]}, \quad K_{12} = \frac{[SL_2][S][L_2]}{[S][L][SL_1]}, \quad K_{13} = \frac{[S][L][SL_1]}{[S][L][SL_1]}
\]

where these constants should be defined in terms of activities.

The formation of a higher complex can directly be written from the substrate and ligand as \( mS + nL \rightarrow S_{m,n}L_n \), and the overall binding constant is defined as

\[
\beta_m = \frac{[S_{m,n}L_n]}{[S]^m[L]^n},
\]

where \( S \) is the substrate, \( L \) is the ligand interacting with the substrate, \( m \) and \( n \) are the stoichiometries of \( S \) and \( L \). The observed equilibrium binding constant \( K_{app} \) or \( K_{obs} \) for drug-DNA interactions can be extracted through construction of Scatchard plots. The concentration of the bound species \( C_b \) is calculated in different ways depending on the method used. The concentration of the free drug \( C_f \) is calculated by using the relationship \( C_f = C_b + C_r \). The term \( r = C_f/[DNA] \) is plotted on the abscissa and \( r/C_f \) is plotted on the ordinate and the \( K_{obs} \) is calculated from the intercept on the ordinate as shown later in this section. Several methods have been used to calculate the apparent binding constants.

Absorbance in the visible region is a convenient tool for detecting complex formation between a dye and DNA. Most small molecules that bind to DNA by intercalation have a visible absorption band by virtue of their heterocyclic structure. As increasing amounts of DNA are added to free dye a shift in \( \lambda_{max} \) to longer wavelength (red shift), and a decrease in absorbance (hypochromic effect) typically occurs. This
presumably occurs due to the interaction of the drug with the electron system of the base pairs. Drugs that do not bind to DNA show insignificant red shifts and hypochromic effect. Sometimes all spectra of a drug-DNA titration pass through a common point, also known as isosbestic point. The most common circumstance under which an isosbestic point is obtained is when there are only two forms of the dye, in the case free in solution and bound. In other words if there is not an isosbestic point, even though the spectra of complex and free dye cross, then there must be more than two forms of the dye. Blake and coworkers discovered that the isosbestic point for the binding of proflavine to DNA disappears at high loads of the drug. This might be due to outside stacking of the drug on the surface of DNA, suggesting two modes of binding. From the UV-visible titration, concentration of the bound drug can be calculated from the expression \( C_b = \frac{\Delta A}{\Delta c} \) where \( \Delta A \) is the change in the absorbance after the addition of the drug and \( \Delta c \) is the difference in the molar extinction coefficient of the free and the bound drug. The Benesi-Hildebrand model as shown in equation (2) is also used at low drug load \( i \) to calculate the binding constants from UV-visible titration data.

\[
\frac{[\text{DNA}]C_i}{\Delta A} = \frac{1}{\Delta c K_{\text{app}}} + \frac{[\text{DNA}]}{\Delta c} \tag{2}
\]

where \( C_i \) is the total drug concentration, [DNA] is the concentration of DNA, and \( \Delta A \) is the difference in the absorbance of the free and the bound drug at a given point. A plot of \([\text{DNA}]C_i / \Delta A \) vs[DNA] has a linear variation with slope \( 1/\Delta c \) and intercept \( 1/K_{\text{app}} \Delta c \). The ratio of the slope to intercept is the binding constant for the process. Equation (2) suffers from the disadvantage that the extrapolation to infinite DNA concentration is not
clear cut, since the equation is linear in [DNA]. Hence a variation of this approach
known as the half reciprocal approach shown in equation (3) is used to calculate $\Delta c$ and
$K_{app}$.

$$\frac{[DNA]}{\Delta c_{app}} = \frac{[DNA]}{\Delta c_{K_{app}}} + \frac{1}{\Delta c} \quad (3)$$

where $\Delta c_{app}$ is the difference between the molar extinction coefficient of the free drug and
the apparent bound drug.\textsuperscript{206}

Frequently, cooperativity is calculated using the Hill model.\textsuperscript{207} The Hill equation is

$$\log \frac{f}{1-f} = n \log (C_r) + \log K \quad (4)$$

where $n$ is the cooperative factor, $f = r/n$ where $r = C/K[DNA]$, $K$ is the apparent binding
constant and $n$ is the binding site size. $n$ values greater than 1 indicate cooperative binding. However, care must be taken in using a Hill model for calculating $K$ as this is a
log-log plot and hence the errors increase in an exponential manner. A direct plot can be
used to calculate binding constants for two distinct sizes where $r$ vs $\log C_r$ is plotted and
fitted to an uncoupled transition model as per the equation:

$$r = \left( \frac{n_1 K_1 C_r}{1 + K_1 C_r} \right) + \left( \frac{n_2 K_2 C_r}{1 + K_2 C_r} \right) \quad (5)$$

where $n_1$, $K_1$, and $n_2$, $K_2$ are the binding site sizes and the binding constants of the two
different binding sites, $C_r$ is the concentration of the free ligand.\textsuperscript{208}
Atomic Force Microscopy

Since atomic force microscopy (AFM) provides the ability to investigate surface structure at nanometer to sub-angstrom resolution in ambient and liquid environments, it has been used routinely over the past two decades in many scientific fields. Indeed, AFM has contributed to groundbreaking research in the investigation of DNA, proteins, and cells in biological studies, structure and component distribution in polymer science, piconewton force interactions and surfactant behavior in colloid science, and physical/mechanical properties and fabrication variables in the material sciences. Pharmaceutical research often consists of a combination of these scientific branches, making it a particularly viable field for the application of AFM. The ability of AFM to provide high-resolution, three-dimensional surface structure, regardless of sample conductivity, makes it a powerful complement to other common analytical techniques currently available.  

AFM is the most commonly used form of the scanning probe microscopy (SPM) family of techniques. The origin of SPM began with the development of the scanning tunneling microscope (STM) by researchers at IBM. The ability of the STM to resolve atomic structure on a sample surface earned the inventors the Nobel Prize in 1986. However, the STM can only be applied to conductive or semiconductive specimens. To broaden this type of microscopy to the study of insulators, the atomic force microscope was developed in collaboration between IBM and Stanford University in 1986. AFM is performed by scanning a sharp tip on the end of a flexible cantilever across a sample surface, while maintaining a small, constant force. The tips typically have an end radius of 5 nm to 10 nm, although this can vary depending on tip type. The
scanning motion is conducted by a piezoelectric tube scanner that scans the tip in a raster pattern with respect to the sample as depicted in Figure 23. The tip-sample interaction is monitored by reflecting a laser off the back of the cantilever onto a split-photodiode detector. The two most commonly used modes of operation are Contact Mode AFM and Tapping Mode AFM, which are conducted in air and liquid environments. In contact mode AFM, a constant cantilever deflection is maintained by a feedback loop that moves the scanner vertically ($z$) at each lateral ($x$, $y$) data point to form the topographic image. By maintaining a constant deflection during scanning, a constant vertical force is maintained between the tip and sample. Applied forces during imaging typically range between 0.1 and 100 nN. Although contact mode has proven useful for a wide range of applications, it sometimes has difficulty on relatively soft samples.212
Figure 23. Components of a Atomic Force Microscopy Instrument.
Tapping Mode AFM consists of oscillating the cantilever at its resonance frequency (typically ~300 kHz) and scanning across the surface with a constant, damped amplitude. The feedback loop maintains a constant root-mean-square (RMS) amplitude by moving the scanner vertically during scanning, which correspondingly maintains a constant applied force to form a topographic image. The advantage of Tapping Mode is that it typically operates with a lower vertical force than that possible with contact mode, and it eliminates the lateral, shear forces that can damage some samples. Thus, Tapping Mode has become the preferred technique for imaging soft, fragile, adhesive, and particulate surfaces. Although the initial use of SPM was to produce high-resolution topographic images, a number of SPM techniques have been developed to study physical and materials properties about sample surfaces. These techniques are commonly used to investigate differences in friction, adhesion, elasticity, hardness, magnetic and electrostatic fields, carrier concentration, conductivity, and temperature distribution. Fundamental force studies are also conducted to study adhesive, attractive, and repulsive interactions between specimens. One of the important innovations that has been integrated into commercial atomic force microscopes is environmental control of the sample. Imaging at elevated temperatures has made it possible to study thermal phase transitions in ambient or inert gas conditions. Imaging in fluid at elevated temperatures has provided the ability to image structures at a physiological temperature of 37°C. Atmospheric hoods are also commonly used in AFM to control humidity or to conduct experiments under specific atmospheric conditions.

Interactions between biological specimens, such as ligand-receptor and protein-DNA systems, have frequently been studied by AFM, and can also be applied to the
study of drug interactions with a variety of biological specimens. For example, AFM-based immunological studies have investigated antibody-antigen binding interactions, and drug-DNA complexes have been studied with AFM to determine DNA ligand mode-of-binding. This is of considerable interest since nucleic acid ligands are commonly used as anticancer drugs and in the treatment of genetic diseases. However, determining whether they bind to DNA by intercalation within major and minor grooves, by "nonclassical" modes, or by a combination of these modes can often be difficult and labor intensive. AFM was used to study drug binding mode, affinity, and exclusion number by comparing the length of DNA fragments that have and have not been exposed to the drug. It is known that if intercalative binding is occurring, the DNA strand increases in length. Furthermore, the degree of lengthening is informative in determining the binding affinity and the site-exclusion number. AFM was shown to be an effective means of seeing and measuring any changes in the DNA strand. When exposed to ethidium, a well characterized intercalator, the DNA strand was shown through AFM to have increased in length from 3300 nm to 5250 nm as indicated by Figure 24.

Similarly, AFM intercalative binding studies showed the increase in the DNA strand, from 3300 nm to 4670 nm, upon exposure to daunomycin, an anticancer drug used to treat leukemia. This technique has also successfully been applied to new drugs in which the mode of binding was unclear. Exposure of 2,5-bis(4-amidinophenyl) (APF), a new drug for the treatment of Pneumocystis carinii pneumonia, did not produce lengthening of the DNA strands, indicating that the drug binds by nonintercalative modes. Although, many AFM studies have concentrated on
acetic acid (in deionized water) \( \lambda_{\text{max}} \) (nm), (log \( \varepsilon \) (dm\(^3\) mol\(^{-1}\) cm\(^{-1}\))): 314 (0.680), 350 (0.680), 354 (0.674), 358 (0.667), 614 (0.752). IR (KBr) \( \nu \) (cm\(^{-1}\)): 2352, 1787, 1696, 1601, 1496, 1393, 1078, 757, 487. Elemental analysis: Calculated for \( \text{C}_{24}\text{H}_{13}\text{N}_{12}\cdot\text{H}_2\text{O} \): C, 62.68; H, 2.98; N, 31.34; Found: C, 62.35; H, 2.44; N, 31.09.

**Tetraethanolpyridino (3.4-b:3'.4'-g:3'''.4'''-l:3'''.4'''-q)porphyrailandium Iodide (2)**

Porphyrinate I (150 mg, 0.289 mmol) was partly dissolved in 50 mL of nitromethane followed by addition of excess 2-iodoethanol (7.70 mL, 0.098 mol). The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. It was observed that the tetra-atomic species formed causes a red shift of the Seret band and reduces the number of Q bands. The reaction was complete after refluxing for 2h. It was cooled to room temperature and the precipitate was isolated by filtration. The green solid obtained was washed with nitromethane (30 mL) and purified by continuous extraction in a Soxhlet apparatus with nitromethane (30 mL), then with hexane (30 mL).

The resulting solid (2) was dried under vacuum to a constant weight (0.228 mmol, 275 mg), (yield 79%). Decomposition temperature > 250 °C. Calculated for \( \text{C}_{36}\text{H}_{14}\text{N}_{12}\cdot\text{I} \): M = 1285; Found: ESI-MS: m/z 697 (M – 4I). UV-visible (de-ionized water) \( \lambda_{\text{max}} \) (nm), (log \( \varepsilon \) (dm\(^3\) mol\(^{-1}\) cm\(^{-1}\))): 347 (2.740), 368 (3.518), 584 (7.026). IR (KBr) \( \nu \) (cm\(^{-1}\)): 3416, 1628, 1478, 1457, 1307, 1225, 1166, 1112, 1068, 1020, 904, 722, 675, 576. Elemental Analysis: Calculated for \( \text{C}_{36}\text{H}_{14}\text{N}_{12}\cdot\text{I} \): C, 35.38; H, 2.88; N, 13.33; Found: C, 35.41; H, 2.53; N, 14.06.

**Manganese Tetraethanolpyridino (3.4-b:3'.4'-g:3'''.4'''-l:3'''.4'''-q) porphyrailandium Iodide (3)**
Porphyrinate 2 (100 mg, 0.0829 mmol) was dissolved in 35 mL of methanol and 5 mL of deionized water. Manganese acetate (22 mg, 0.0912 mmol) was added to the solution. The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 6 hours. The reaction mixture was allowed to cool to room temperature, filtered (no residue) and concentrated under reduced pressure. The resulting green solid obtained was washed with 2-propanol (30 mL) and purified by continuous extraction in a Soxhlet apparatus with 2-propanol (30 mL). The resulting solid (3) was then dried under vacuum to a constant weight: (0.0651 mmol, 82 mg), (yield 78%). Decomposition temperature > 270 °C. Calculated for \( \text{C}_9\text{H}_2\text{N}_2\text{O}_4\text{MnL}_4 \): M = 1259. Found: ESI-MS m/z 751 (M – 4I) - . UV-visible (dionized water) \( \lambda_{max} \) (nm), (log e (dm\(^3\) mol\(^{-1}\) cm\(^{-1}\))): 225 (209.48), 368(45.02), 620 (84.90). IR (KBr) ν (cm\(^{-1}\)): 3384, 3000, 1624, 1473, 1422, 1304, 1168, 1135, 1066, 833, 773, 720, 668, 528, 424. Elemental analysis: Calculated for \( \text{C}_9\text{H}_2\text{N}_2\text{O}_4\text{MnL}_4 \): C, 34.30; H, 2.54; N, 13.07. Found: C, 34.45; H, 2.55; N, 13.07.

**Cobalt Tetraethanolporphyrine (3,4-b:3',4'-g:3''-h:3'''-q) porphyrizinium iodide (4)**

Porphyrinate 2 (100 mg, 0.0829 mmol) was dissolved in 35 mL of methanol and 5 mL of deionized water. Cobalt acetate (22 mg, 0.0912 mmol) was added to the solution. The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 7 hours. The reaction mixture was allowed to cool, filtered (no residue) and concentrated under reduced pressure. The resulting green solid obtained was washed with 2-propanol (30 mL) and purified by
continuous extraction in a Soxhlet apparatus with 2-propanol (30 mL). The resulting solid (4) was then dried under vacuum to a constant weight: (0.08619 mmol, 782 mg) was obtained, (yield 79%). Decomposition temperature > 285 °C. Calculated for 
\[ \text{C}_{28}\text{H}_{22}\text{N}_{12}\text{O}_{8}\text{Cl}_{4} \cdot \text{C}_{4} \] 
M = 1263; Found: ESI-MS: m/z 755(M – 4I)^+. UV-visible (deionized water) \( \lambda_{\text{max}} \) (nm), (log ε (dm^3 mol^{-1} cm^{-1})): 351 (5.271), 368 (5.895), 659 (8.610). IR (KBr) ν (cm^{-1}): 3384, 3000, 1624, 1473, 1422, 1394, 1168, 1135, 1066, 833, 773, 720, 668, 528, 424. Elemental analysis: Calculated for 
\[ \text{C}_{28}\text{H}_{22}\text{N}_{12}\text{O}_{8}\text{Cl}_{4} \cdot \text{C} \] 
C, 34.19; H, 2.53; N, 13.30; Found: C, 34.45; H, 2.53; N, 13.33.

Copper Tetraethanolpyridino (3,4-b:3',4'-g:3'':4'':-l:3''',4''''-q) porphyrizinium Iodide (5)

Porphyrazine 2 (150 mg, 0.124 mmol) was dissolved in 35 mL of methanol and 5 mL of deionized water. Copper acetate (27 mg, 0.136 mmol) was added to the solution. The mixture was slowly heated over 1 h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 8 hours. The reaction mixture was allowed to cool to room temperature, filtered (no residue), and concentrated under reduced pressure. The resulting blue solid obtained was washed with 2-propanol (50 mL) and purified by continuous extraction in a Soxhlet apparatus with 2-propanol (30 mL). The resulting solid was then dried under vacuum to a constant weight to give 109 mg (0.0859 mmol) of (5), (yield 69%). Decomposition temperature > 295 °C. Calculated for 
\[ \text{C}_{28}\text{H}_{22}\text{N}_{12}\text{O}_{8}\text{Cl}_{4} \] 
M = 1257. Found: MALDI-TOF-MS: m/z 759.20 (M- 4I)^+, 716.82 (M- 4I- \( \text{CH}_3\text{CH}_2\text{O} \))\(^{+}\), 671.68 (M – 4I – 2\( \text{CH}_3\text{CH}_2\text{OH} \))\(^{+}\), 626.71 (M – 4I – 3\( \text{CH}_3\text{CH}_2\text{OH} \))\(^{+}\), 581.76 (M- 4I – 4\( \text{CH}_3\text{CH}_2\text{OH} \))\(^{+}\); ESI-MS: m/z 760(M – 4I)^+. UV-
visible (deionized water) $\lambda_{\text{max}}$ (nm), (log $e$ (dm$^3$ mol$^{-1}$ cm$^{-1}$)): 259 (209.50), 368 (45.024), 620 (84.898). IR (KBr) $\nu$ (cm$^{-1}$): 3384, 3000, 1624, 1473, 1422, 1304, 1168, 1135, 1066, 833, 773, 720, 668, 528, 424. Elemental analysis: Calculated for $\text{C}_{34}\text{H}_{32}\text{N}_{12}\text{O}_{4}\text{ZnI}_{4}$. C, 34.07; H, 2.52; N, 13.25; Found: C, 34.82; H, 2.44; N, 13.10.

**Zinc Tetrathiaalopyridine (3,4-b:3',4'-g:3",4":5'"-q) porphyrizinium Iodide (6)**

Porphyrinone 2 (100 mg, 0.0829 mmol) was partly dissolved in 35 mL of methanol, and 5 mL deionized water. Zinc acetate (182 mg, 0.9911 mmol) was added to the solution. The mixture was slowly heated over 1 h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 8 hours. The reaction mixture was allowed to cool to room temperature, filtered (no residue) and concentrated under reduced pressure. The dark green solid obtained was washed with 2-propanol (30 mL) and purified by continuous extraction in a Soxhlet apparatus with nitroethane (30 mL).

The resulting solid obtained was then dried under vacuum to a constant weight to give 760 mg (0.0598 mmol) of 6, (yield 72%). Decomposition temperature > 296 °C. Calculated for $\text{C}_{34}\text{H}_{32}\text{N}_{12}\text{O}_{4}\text{ZnI}_{4}$. M = 1270. Found: MALDI-TOF-MS: $m/z$ 750.36 (M - 4I)$^+$, 716 (M - 4I - CH$_2$CH$_2$OH)$^+$, 671.02 (M - 4I - 2CH$_2$CH$_2$OH)$^+$, 626 (M - 4I - 3CH$_2$CH$_2$OH)$^+$; ESI-MS: $m/z$ 762 (M - 4I)$^+$. UV-visible (deionized water) $\lambda_{\text{max}}$ (nm), (log $e$ (dm$^3$ mol$^{-1}$ cm$^{-1}$)): 379 (31.38), 608 (19.99), 657 (67.50), 670 (73.71). IR (KBR) $\nu$ (cm$^{-1}$): 3731, 3419, 3000, 2272, 1631, 1478, 1300, 1166, 1109, 1072, 713, 672, 653. Elemental analysis: Calculated for $\text{C}_{34}\text{H}_{32}\text{N}_{12}\text{O}_{4}\text{ZnI}_{4}$. C, 34.95; H, 2.54; N, 13.23; Found: C, 34.38; H, 2.53, N, 13.19.
Manganese (II) 4, 4', 4",4""""-tetraaza-2H, 3H-phthalocyanine (7).

3,4-pyridinedicarbonitrile (506 mg, 3.7 mmol) was dissolved in 60 mL of 2-(dimethylamino)ethanol. Manganese acetate (226 mg, 0.925 mmol) was added to the solution. The mixture was slowly heated over 1 h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 72 hours. The reaction mixture was allowed to cool to room temperature, filtered, and the resulting green solid obtained was washed with 2-(dimethylamino)ethanol (25 mL) and purified by continuous extraction for 24 hours in a Soxhlet apparatus with hexane (30 mL), followed by continuous extraction with acetone (30 mL). The resulting solid was then dried under vacuum to a constant weight to afford 521 mg (0.912 mmol) of 7 at (yield
98%). Decomposition temperature > 270 °C. IR (KBr) ν (cm⁻¹): 3421, 3070, 2944, 2576, 2227, 1168, 1342, 1165, 1021, 953, 780, 672, 654. Elemental analysis: Calculated for C₃₈H₃₂N₃₂MnO₆·3.5 H₂O: C, 53.35; H, 2.97; N, 25.90; Found: C, 53.08; H, 3.00; N, 26.54.

**Manganese Tetraethanolpyridino (3,4-b:3',4'-g:3''-h:3''':4'''-q) porphyrazinium Iodide (8).**

Manganese (II) 4, 4', 4'', 4'''-tetraaza-29H, 31H-phthalocyanine 7 (300 mg, 0.275 mmol) was partly dissolved in 40 mL of nitromethane followed by addition of excess 2-iodoethanol (7.13 mL, 65 mmol). The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. It was observed that the tetracatonic quaerontized species formed because of a red shift of the Soré band. The reaction was complete after refluxing for 24h. The reaction mixture was allowed to cool to room temperature and the precipitate was isolated by filtration. The green solid obtained was washed with nitromethane (30 mL) and purified by continuous extraction in a Soxhlet apparatus with nitromethane (30 mL), then hexane (30 mL). The resulting solid was dried under vacuum to a constant weight to afford 175 mg (0.228 mmol) of 8, (yield 51%).

Calculated for C₃₈H₃₂N₃₂O₆·Mn·H₂O: M = 1259. Found: ESI-MS: m/z 751 (M+ - 4I)

UV-visible (dionized water) λ max (nm), (log ε (dm³ mol⁻¹ cm⁻¹)): 225 (209.48), 368(45.02), 620 (84.90). IR (KBr) ν (cm⁻¹): 3384, 3000, 1624, 1473, 1422, 1304, 1168, 1135, 1066, 873, 773, 720, 668, 528, 424. Elemental analysis: Calculated for C₃₈H₃₂N₃₂O₆Mn: C, 34.30; H, 2.54; N, 13.07; Found: C, 34.40; H, 2.45; N, 13.91.
Cobalt (II) 4', 4",4'"-tetraaza-29H, 31H-phthalocyanine (9).

3,4-pyridinedicarbonitrile (500 mg, 3.7 mmol) was dissolved in 60 mL of 2-(dimethylamino)ethanol. Cobalt acetate (231 mg, 3.929 mmol) was added to the solution. The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 72 hours. The reaction mixture was allowed to cool, and filtered. The resulting green solid obtained was washed with 2-(dimethylamino)ethanol (25 mL) and purified by continuous extraction in a Soxhlet apparatus for 24 hours with hexane (25 mL), followed by continuous extraction with acetone (25 mL). The resulting solid was then dried under vacuum to a constant weight to afford 485 mg (0.0619 mmol) of 9, (yield 91%). Decomposition temperature > 285 °C. IR (KBR) ν (cm⁻¹): 3442, 3090, 2933, 2588, 2227,1951, 1607, 1021, 976, 786, 672, 651. Elemental analysis: Calculated for C₃₈H₃₂N₂Co 3.5H₂O: C, 53.38; H, 3.20; N, 25.59; Found: C, 53.71; H, 3.58; N, 25.09.

Cobalt Tetraethanolpyridino (3,4-b:3'\',4"-q:3":4":l:3":4":q) perphyrazinium Iodide (10)

Cobalt (II) 4', 4",4'"-tetraaza-29H, 31H-phthalocyanine 9 (200 mg, 0.347 mmol) was partly dissolved in 50 mL of nitromethane followed by addition of excess iodoethanol (8.15 mL, 116 mmol). The mixture was slowly heated in 1h to reflux. The reaction was monitored by UV-visible spectroscopy. It was observed that the tetracationic quaternized species formed causing a red shift of the Soret band. The reaction was complete after refluxing for 24h. The reaction mixture was allowed to cool to room temperature and the precipitate was isolated by filtration. The green solid obtained was washed with
nitromethane (30 mL) and purified by continuous extraction in a Soxhlet apparatus with nitromethane (30 mL), hexane (30 mL). The resulting solid was dried under vacuum to a constant weight: 8.254 mmol 10 (322 mg) was obtained (yield 73.5%).

Calculated for Cu₅H₂N₃O₅Co₄. M = 1263; Found: ESL-MS: m/z 755(M - 4I)⁺. UV-visible (deionized water) λ_max (nm), (log ε (dm³ mol⁻¹ cm⁻¹)): 251 (5.271), 368 (5.895), 659 (8.610). IR (KBr) ν (cm⁻¹): 3384, 3000, 1624, 1473, 1422, 1304, 1168, 1135, 1066, 833, 773, 720, 668, 528, 424. Elemental analysis: Calculated for Cu₅H₂N₃O₅Co₄: C, 34.19; H, 2.53; N, 13.30; Found: C, 34.20; H, 2.48; N, 13.11.

Copper Tetraethanolpyridino (3,4-b:3',4'-g:3''-e:3'''-l:3''''-q) porphyrizinium Iodide (12).

Copper (II) 4, 4', 4'',4'''-tetraaza-29H, 31H-phthalocyanine 11 (100 mg, 0.170 mmol) was partly dissolved in 30 mL of nitromethane followed by addition of excess 2-iodoethanol (6.36 mL, 58.9 mmol). The mixture was slowly heated in 1h to reflux. The reaction was monitored by UV-visible spectroscopy. It is observed that the tetracationic quarterized species formed causes a red shift of the Soret band. The reaction was complete after refluxing for 48h. The reaction mixture was allowed to cool to room temperature and the precipitate was isolated by filtration. The green solid obtained was washed with nitromethane (10 mL) and purified by continuous extraction in a Soxhlet apparatus with nitromethane (30 mL), followed by hexane (30 mL); The resulting solid was dried under vacuum to a constant weight to afford 215 mg (0.169 mmol) of 12, (yield 69.59%). Decomposition temperature > 295 °C. Calculated for Cu₅H₂N₃O₅Cu₄. M = 1267. Found: MALDI-TGF-MS: m/z 759.20 (M - 4I)⁺, 716.82 (M - 4I- CH₃CH₂OH)⁺.
Zinc (II) 4, 4', 4'', 4'''-tetraaza-29H, 31H-phthalocyanine (13).

3,4-pyridinedicarbonitrile (1.0 gram, 7.4 mmol) was dissolved in 60 mL of 2-(dimethylamino)ethanol. Zinc acetate (331 mg, 1.85 mmol) was added to the solution. The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. The reaction was complete after refluxing for 72 hours. The reaction mixture was allowed to cool, and filtered. The resulting green solid obtained was washed with 2-(dimethylamino)ethanol (50 mL) and purified by continuous extraction in a Soxhlet apparatus for 24 hours with hexane (50 mL), followed by continuous extraction with acetone (50 mL). The resulting solid was then dried under vacuum to a constant weight to afford 31/2 mg (0.0619 mmol) of 13, (yield 80.0%). Decomposition temperature > 290 °C. IR (KBr) ν (cm⁻¹): 3421, 3070, 2944, 2576, 2227, 1168, 1342, 1165, 1116, 1057, 903, 836, 653. Elemental analysis. Calculated for C₉₆H₃₉N₂₇Zn: C, 57.60; H, 2.06; N, 28.90; Found: C, 57.32; H, 1.96; N, 28.71.
Zinc Tetraethanopyridino (3,4-b:3',4'-g;3',4''-l:3‴,4‴-q) porphyrizinium Iodide (14).

Zinc (II) 4, 4', 4'',4'''-tetraaza-29H, 31H-phthalocyanine 10 (100 mg, 0.170 mmol) was partly dissolved in 60 mL of nitromethane followed by addition of excess 2-iidoethanol (9.54 mL, 87.2 mmol). The mixture was slowly heated over 1h to reflux. The reaction was monitored by UV-visible spectroscopy. It was observed that the tetracationic quaternized species formed causes a red shift of the Soret band. The reaction was complete after refluxing for 48h. The reaction mixture was allowed to cool to room temperature and the precipitate was isolated by filtration. The green solid obtained was washed with nitromethane (30 mL) and purified by continuous extraction in a Soxhlet apparatus with nitroethane (30 mL), followed by hexane (30 mL). The resulting solid was dried under vacuum to a constant weight to afford 100 mg (0.169 mmol) of 14, (yield 50.0%). Decomposition temperature > 295 °C. Calculated for C_{36}H_{32}N_{12}O_{4}ZnI_{4}: M = 1270, Found: MALDI-TOF-MS: m/z 766.30 (M - 4I)^−, 716 (M - 4I - CH_{3}CH_{2}OH)^−, 671.92 (M - 4I - 2CH_{3}CH_{2}OH)^−, 626 (M - 4I - 3CH_{3}CH_{2}OH)^−; ESI-MS: m/z 762 (M - 4I)^−. UV-visible (deionized water) λ_{max} (nm), (log ε (dm³ mol⁻¹ cm⁻¹)): 379 (31.38), 508 (19.99), 657 (67.50), 670 (73.73). IR (KBR) ν (cm⁻¹): 3731, 3419, 3009, 2272, 1631, 1478, 1300, 1156, 1109, 1072, 713, 672, 653. Elemental analysis: Calculated for C_{36}P_{3}N_{12}O_{4}ZnI_{4}: C, 34.05; H, 2.54; N, 13.23; Found: C, 34.45; H, 2.50; N, 13.46.
Absorption spectra were recorded at 25.0 ± 0.1 °C using a Cary 100 UV/VIS spectrophotometer fitted with a constant temperature accessory and interfaced with a computer for data collection. Titration experiments for binding were performed in K-BPES buffers with 200 mM K⁺ and/or 20 mM Mg²⁺ for d(T₃G₃), and 100 mM K⁺ for the aptamer. Optical monitoring was done at the respective λₘₐₓ for the Soret band of each porphyrinate. Solutions of fixed concentration of porphyrizines (3-5 µM) were titrated by stepwise addition of aliquots of DNA solution containing the porphyrizine at the same concentration (30-50 µM in quadruplex or duplex). After each addition, the solution was incubated for 5 minutes before any readings were taken. All of the titrations were done at 25 °C. Titration data was plotted and transferred to the program Power Point. Titration data were then cast into the form of Scatchard plots of n/Cᵣ versus r for analysis. Data were fit through simple Scatchard equation:

\[ r/Cᵣ = K(n-r) \]  

(6)

where K is the equilibrium binding constant and n represents the number of ligand molecules bound per DNA quadruplex. The binding ratio r is defined as Cᵣ/[DNA], where the molar concentration of bound porphyrin, Cᵣ, is equal to ΔA /Δε (ΔA is the difference in absorption of the free porphyrin and porphyrin in the presence of DNA at respective λₘₐₓ of each porphyrin and Δε is the difference in molar extinction coefficients between the free and bound porphyrin, ε₁ - ε₂). The concentration of free porphyrin, Cᵓ, was calculated using Cᵓ = C₀ + Cᵣ where C₀ is the total concentration of porphyrin. The DNA concentration is expressed in terms of the molar concentration per quadruplex.
These terms can also be determined by calculating first the fraction of bound drug \( a \) as given directly by the relative \( \Delta A \) hypochromicity term using \( \Delta A = (A_{\text{free}} - A)/(A_{\text{free}}/A_{\text{tot}}) \), where \( A_{\text{free}} \) and \( A_{\text{tot}} \) are the absorbances for free and fully bound ligands at respective \( \lambda_{\text{max}} \) of each porphyrin. The term \( C_t \) is calculated by \( C_t = (1 - a) \). \( C_t \) and binding ratio \( r \) are defined as \( (C - C_t)/[\text{DNA}] \). No difference was found in the observed Scatchard plots obtained by the two methods. Nonlinear least squares regression, fit line was plotted for all the fits and error bars (5% of the observed Y value) were added to maintain a 95% confidence interval.

**Atomic Force Microscopy (AFM) Experiments**

DNA samples for imaging were prepared by diluting 2.5 μg aliquots of linearized plasmid PRs-316 in water. The samples were denatured by boiling, followed by rapid cooling and lyophilization. The lyophilized samples were then dissolved in the appropriate buffer and incubated at 37 °C for 24 h. After incubation, the samples were diluted 1:100 in 10mM Tris-HCl, pH 7.0, 1mM MgCl₂, and 10 μl was deposited onto a freshly cleaved mica surface. The DNA sample was allowed to absorb for 5 min, washed with 1 mL sterile water and rapidly dried in a stream of N₂ gas. The same protocol was adapted to image DNA in the presence of MTEPPi such that the [MTEPPi]/[DNA] = 2:1. Porphyrin zinc solutions were made in the same buffer as DNA. Imaging was carried out using a commercial ambient scanning probe microscope (Park Scientific Inst., Sunnyvale, CA), employing Au-coated Si₃N₄ cantilevers with sharpened pyramidal tips. The images were recorded in the height and force imaging modes. Additional image analysis performed on an Intel 500 MHz Pentium III-based computer using the UTHSCSA Image
Tool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from mhxrad6.uthscsa.edu).

**Competition Dialysis experiments**

For each competition dialysis assay, 400 mL of the dialysate solution containing 1 uM ligand (Drug) was placed into a 1 liter beaker. A volume of 180 uL of each of the nucleic acid samples was pipetted into a separate 0.5 mL SpectroPor DuploDialyze unit with a 2000 molecular weight cut off. All the oligonucleotides (Table 1) were at the same concentration of 75 uM. The nucleic acid concentration was expressed in terms of the monomeric unit for each polymer. All 10 dialysis units were placed in the beaker containing the dialysate solution. The beaker was covered with a glass covering and wrapped in aluminum foil, kept away from light, and allowed to equilibrate with stirring for 24 hours at room temperature (25 °C) in a temperature controlled bath. After completing the equilibration time, the oligonucleotide samples were removed to microfuge tubes and were diluted to a final concentration of 1% (w/v) with sodium dodecyl sulfate (SDS) by the addition of the appropriate volumes of a 10% (w/v) stock solution. For each separate ligand studied, the total concentration of TEPPi (7026 M⁻¹ cm¹, 684 nm), MnTEPPi (84,900 M⁻¹ cm¹, 620 nm), CoTEPPi (8917 M⁻¹ cm¹, 659nm), CuTEPPi (84,898 M⁻¹ cm¹, 621 nm), ZnTEPPi (73,730 M⁻¹ cm¹, 670 nm), within each dialysis unit was then determined spectrophotometrically (Varian, Cary 100) using each extinction coefficient at the specified wavelength. An appropriate correction for the slight
dilution of the sample resulting from the addition of the stock SDS solution was made. The free ligand concentration ($C_f$) was determined spectrophotometrically using an aliquot of the dialysate solution. The concentration did not change from the initial 1 µM concentration. The amount of bound drug ($C_b$) was determined by the difference of:

$$C_b = C_s - C_f.$$ 

The data was plotted as a bar graph using Harvard Graphics. The apparent binding constants were calculated using the following equation:

$$K_{app} = \frac{C_b}{C_f} \times (\text{NA}_{\text{total}}).$$

Where $C_b$ and $C_f$ (1 µM) are the bound and free ligand concentrations, respectively, and (NA)$_{total}$ (75 µM) is the nucleic acid concentration.
<table>
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<th>Conformation</th>
<th>DNA/oligo</th>
<th>λ (nm)</th>
<th>ε *</th>
<th>Tm (°C)</th>
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<td>Single-stranded</td>
<td>Poly (da)</td>
<td>257</td>
<td>8,600</td>
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<tr>
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<td>257</td>
<td>14,330</td>
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</tbody>
</table>

Table 1. DNA oligonucleotide physical properties used for competitive dialysis study. * ε = (dm^3·mol⁻¹·cm⁻¹)
Porphyrazines synthesis:

A porphyrazine and a series of tetraazatic porphyrazines, unmetallated and metallated were synthesized as described in detail in Chapter III under Materials and depicted below in Scheme I.

Reaction Scheme I:

The first part of our design was to build porphyrazine (1) with nitrogen in its peripherals for reaction with iodoethanol while quaternizing the ring system at the same time. This was accomplished by reacting 3,4-dicyanopyridine with 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) in anhydrous octanol at 185 °C for 2 hours, followed by purification to obtain the neutral tetrapyridino porphyrazine (1) which was obtained in a yield of 45%. The
interactions at the molecular level, responses of living cells have also been visualized.\textsuperscript{219}
Since cells can be imaged in a physiological solution at 37°C, it is possible to monitor the interaction between a metabolically active cell and a chemical or biological additive to the solution environment. Work has been published on observing the interaction between living cells and drugs, viruses, and other chemicals. Rotch and Radmacher conducted "force mapping" studies on 3T3 and NRK fibroblast cells to visualize the effects of various drugs.\textsuperscript{219} Force mapping, also referred to as "the force-volume technique," conducts a lateral array of force curves across a sample surface that results in a force-interaction image. Collecting force curves consists of recording the change in the cantilever deflection as it moves vertically toward the sample until it touches the surface, and then pulls away from the surface and retracts back to its starting position. Force curves can detect repulsive, attractive, and adhesive interactions in the pico-Newton-to-nanoNewton range.\textsuperscript{211}
AFM is also commonly used to gain a better understanding of the physiological mechanisms associated with disease. Some examples of this include AFM studies associated with Alzheimer’s disease, Parkinson’s disease, diabetes, pancreatitis, and cancer. One of the advantages in using an atomic force microscope for this type of work is its ability to perform these investigations in situ. Yip and McLaurin used AFM to study the mechanisms of amyloid-β (Aβ) fibrillogenesis, which plays a role in Alzheimer’s disease. In situ Tapping Mode imaging was performed on total brain lipid bilayers to study the role of membrane composition and peptide structure. Brain lipid bilayers were deposited onto mica and imaged in phosphate-buffer solution (PBS). After introducing the monomeric Aβ peptides into the buffer solution, Aβ1-40 molecules were found to be partially inserted into the bilayer surface. After 15 hours, fibril growth was initiated from these sites, resulting in membrane disruption. To study the specificity of lipid bilayer composition and Aβ sequence, the same experiment was conducted with DMPC bilayers and Aβ1-28 peptides. From these studies it was determined that the fibril formation occurs in the presence of acidic lipids, and that the peptide requires the hydrophobic C-terminal domain, which is critical for anchoring to the lipid to induce fibrillogenesis. Without these critical requirements, membrane disruption is produced by the formation of Aβ aggregate without evidence of fibril formation. Based on these AFM observations, a better understanding of the mechanisms that result in the Aβ fibrillogenesis was formed.

Atomic force microscopy (AFM) has many uses in the field of pharmaceutical research, including the investigation of in situ processes, interaction mechanisms, behavioral properties, and structure-function relationships. Although these examples are
We have discovered a novel one-step, self-assembly synthesis of unmetallized porphyrazines. In contrast to the previously published methods, our approach to the synthesis of tetracationic metal-free and metal containing porphyrazines involves the treatment of 3,4-dicyanopyridine with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in anhydrous octanol at 185 °C for 2 hours. To obtain a tetracationic metal-free porphyrazine, the resultant porphyrazine is treated with an excess of 2-iodoethanol in the presence of niromethane at 101 °C for a period of 24 hours. The tetracationic species thus obtained is dissolved in methanol and water and reacted under reflux with the corresponding metal acetate. We also prepared the same porphyrazines by reacting 3,4-dicyanopyridine with the corresponding metal acetate, followed by reacting the metal porphyrazine obtained with 2-iodoethanol in the presence of niromethane at 101 °C for a period of 24 hours, followed by cooling, filtration and removal of any impurities by soxhlet extraction.
Experimental:

Tetrapyridino (3,4-b:3',4'-g:3''3",4",d:3":4":q)porphyrizene (1)

3,4-pyridinedicarbonitrile (500 mg, 3.8 mmol) was mixed in anhydrous octanol (25 mL), followed by the addition of mmol DBU (2.36 g, 15.2 mmol). The mixture was slowly heated over 40 minutes to reflux (185 °C) and held for an additional 2h. The mixture was allowed to cool to room temperature and the precipitate was isolated by filtration.

The violet solid obtained was washed with octanol (30 mL) and purified by continuous extraction in a Soxhlet apparatus with hexane (30 mL), then aceton (30 mL), and finally, deionized water (30 mL). The resulting solid (1) was dried under vacuum to a constant weight: 0.345 mmol, 225 mg, (yield 45%).

Decomposition temperature > 270 °C. Calculated for C_{28}H_{44}N_{12}·M = 518. Found: MALDI-TOF-MS: m/z 520 (M + 2H)^+; ESI-MS: m/z 515 (M + H)^+. UV-visible (10%)
tetracyclic nonmetallated tetraethanol tetrapyridine porphyrazinium iodide (2) was synthesized by reacting the neutral porphyrazine (1) with a large excess of 2-iodoethanol in the presence of nitromethane. After purification a yield of 79% was obtained. The nonmetallated tetraethanol tetrapyridine porphyrazinium iodide (2) was then metallated with the specific metal acetate in a solution of water/methanol solution at reflux. After purification, the metallated porphyrazines obtained were Mn2+ (3), Cu2+ (4), Co2+ (5), Zn2+ (6) with yields of 78%, 69%, 75% and 72%, respectively. In our second approach to obtaining metallated porphyrazines, we followed the following reaction Scheme II, shown below.

Reaction Scheme II:

The part of the synthesis was based on the synthesis of phthalocyanines, by substituting phthalonitrile with 3,4-dicyanopyridine and reaction in the presence of diethyl ether amine and the corresponding metal acetate at reflux, followed by purification to directly obtain the metallated porphyrazines. (Mn2+ (7), Cu2+ (11), Co2+ (9), Zn2+ (13). The metallated porphyrazines were quaternized with a large excess of iodoethanol in the presence of nitromethane at reflux. Purification led to the metallated porphyrazines (Mn2+ (7), Cu2+ (11), Co2+ (9), Zn2+ (13), with yields of 31%, 69%, 75% and 50%, 101
respectively. The objective for synthesizing these porphyrines was to demonstrate their utility as potential DNA binding agents.

**UV/Visible spectra of porphyrines:**

A great interest has been shown for compounds that have absorb light at long wavelengths. Recently 2,3-naphthalocyanines, especially the tetrazeno-annelated phthalocyanines, have been proposed as photodynamic therapeutic agents. The water solubility of phthalocyanines depends on the type of substituents of the ligand on the phthalocyanine core. In our laboratory, the porphyrines were made water soluble by quaternizing with specific ligands. The advantage of these molecules is that they are very water soluble, making them attractive for carrying them through the cell membrane. The porphyrines that were synthesized in our laboratory show great promise as photosensitizers because these compounds have intense absorptions between 600 -700 nm with extinction coefficients greater than 10^6 L mol^-1 cm^-1 (See Figures 28, 30, 32, 34, 36). Terpyridyl porphyrines exhibit the lowest extinction coefficient when quaternized with acetic acid. (see Figure 38). By Incorporating ligands into this system leading to enhanced water solubility, we were also able to increase the molar absorptivity and obtain higher molar extinction coefficients. Figure 27 depicts the visible absorption spectra of tetraethanolterpyridino porphyrine iodide (TEPPI) indicating absorbances at very low molar concentrations of 4.66 X 10^-4 M. Another physical property that makes these porphyrines attractive as photodynamic therapeutic agents is the fact that they have large molar extinction coefficients even at low molar concentration. (See Figure 28).
Upon complexation of the ring with manganese, a divalent metal, the ring system becomes contracted by the bonds and in effect changes the molar absorptivity of the ring system as depicted in Figure 29. Further, comparing a nonmetallated system (TEPPI) with (MnTEPPI) one can note that the absorbance shifts by 40-50 nm to a lower wavelength when incorporating manganese into the ring system. When the metal is incorporated into the ring system the molar extinction value goes up significantly as indicated by Table 2. The spectral changes could be due to the partial charge transfer from the nitrogen atom to the porphyrazine π-electron system. Additionally, the spectral changes may also be due to the fact that porphyrizines exist in solution as nonequivalent tautomers arising from the delocalization of the electrons.

The chemical changes that take place when incorporating a metal center, allows one to control the optical absorption, ionization and the redox potentials of the molecule. The quaternization of the porphyrizines also allows for many changes in aqueous systems. The interaction between water and the charged species also affects the electronic nature of the molecule as reflected in Figures 27, 29, 31, 33 and 35.
Figure 27. UV/Visible absorbance spectra of TEPPI in water.

Figure 28. Absorbance versus moles/liter of TEPPI.
Figure 29. UV/VIS absorbance spectra of MnTEPP in water.

Figure 30. Absorbance versus moles/liter of MnTEPP.
As noted above, the insertion of the divalent metal Mn decreases the wavelength of maximum absorbance ($\lambda_{\text{max}}$) and increases the extinction coefficient of the porphyrazine. Examination of the data in Figures 31-38 and in Table 1, shows how the nature of the metal influences the magnitude of the spectral changes. Changing from manganese to cobalt shifts $\lambda_{\text{max}}$ to a higher wavelength as depicted in Figure 31 and increases the extinction coefficient. As can be seen from Figure 33 and Figure 35, there is a shift by 10 nm in the wavelength when substituting copper for zinc.
Figure 31. UV/VIS absorbance spectra of CoTEPPI in water.

Figure 32. Absorbance versus moles/liter of CoTEPPI.
Figure 33. UV/VIS absorbance spectra of CuTEPPi in water.

Figure 34. Absorbance versus moles/liter of CuTEPPi.
Figure 35. UV/VIS absorbance spectra of ZnTEPPI in water.

Figure 36. Absorbance versus moles/liter of ZnTEPPI.
**Figure 37.** UV/VIS absorbance spectra of TPP in water.

**Figure 38.** Absorbance versus moles/liter of TPP.
Table 2. Electronic spectral data of the porphyrines in deionized water.

(E = dm·mol⁻¹·cm⁻¹).

* In 10% acetic acid.

Our findings indicate that symmetrically substituted metallated or nonmetallated porphyrines are readily available by the synthetic methods developed in our laboratory. These compounds are characterized by long wavelength absorption with high molar extinction coefficients values as indicated by Table 2. The solubility of these quaternized porphyrines in water also makes them better candidates for being able to move through the cell membrane in carrier systems like PEG. The viability of having excellent singlet oxygen quantum yields and good photosensitizing stability is also another property that makes these compounds potential photodynamic therapeutic agents. Further, these compounds are unique, because even at low concentrations, they still exhibit large absorption. This physical
property allows one to use the compounds in very low concentration levels when targeting tumor cells and also minimize cytotoxic effects.

Titration of DNA oligonucleotides with porphyrines:

Titration of DNA oligonucleotide with the above metallated and unmetallated porphyrines was monitored by visible absorption spectroscopy. The main focus of these studies was to observe any changes of the Soret band of the porphyrine in the presence of excess DNA. The spectra are characterized by orange or red shifts and hypochromicities or hyperchromicities of the Soret band. The changes in the Soret band of the porphyrine indicate the type of interaction within a ligand-DNA complex. The degree of hypochromicity or hyperchromicity and extent of shift in λmax indicates the types of interactions that take place such as intercalation, outside binding or outside stacking. As indicated by Figure 69 one can see the percent degree of hypochromicity or hyperchromicity due to the ligand-DNA interaction. This is followed by the un/visible spectra of each TEPPI, ZnTEPPI and CoTEPPI interactions with different DNA oligonucleotides.

As can be shown from Figures 39-68, porphyrines bind to DNA duplexes and DNA quadruplexes. Figure 69 clearly shows the degree that porphyrines bind. The degree and the way in which they bind can be determined by the degree of hypochromicity or hyperchromicity. The titration curves obtained as a result of the interaction of TEPPI, ZnTEPPI and CoTEPPI with calf thymus are depicted in Figures 39, 50, and 59 respectively. All of the curves show significant hypochromicity (decrease in absorbance) as well as a slight red shift in the λmax of respective porphyrines, although the magnitudes vary in different
cases. In addition, an isorhectic point is observed in most of the curves which is indicative of a two state system (free and fully bound).
Interaction of TEPPi with calf thymus:

**Figure 39.** UV/VIS spectra for the titration of TEPPi in the presence of calf thymus.

**Figure 40.** UV/VIS spectra of calf thymus + TEPPi fully bound and TEPPi unbound.
Figure 39 shows that as a solution of porphyrin and calf thymus DNA reach saturation when added into a porphyrin solution at a constant porphyrin concentration, the absorption bands become indistinguishable from each other and that the absorption begins to go up instead of continually going down. This phenomenon is probably due to other electrostatic binding forces taking place. As depicted in Figure 40 the absorption of the fully bound calf thymus DNA with TEPPi shows a higher absorption band than the free TEPPi. This phenomenon could be due to the following: as the calf thymus DNA becomes saturated with TEPPi, the interactions that then take place are due to outside stacking and electrostatic interactions in the minor groove.

As depicted in Figure 41, one can note that the absorption decreases without much shifting of the Soret band to lower or higher wavelength. This can be explained by the fact that only electrostatic interactions are taking place. It can also be noted that the absorption band is not sharp and that it tends to broaden as it reaches saturation.

Figure 42 shows the titration of TEPPi (free) versus TEPPi + d(T3G3). As can be noted, the absorbance and wavelength increases and the absorbance remains broad. This phenomena could be due to the electrostatic interactions taking place and also due to complete saturation of the ligand around the major and minor grooves of the DNA (quadruplexes do not have grooves as such).

When either zinc or cobalt is incorporated into the ring system as depicted in Figure 51 and 61 respectively and titrated in the presence of d(T3G3), a similar pattern is obtained and characterized by a broad absorption band followed by shifting to higher wavelength in the red region. When the titration is conducted in the presence of d(G7T7G7), as depicted by Figures 43, 53 and 63 and going from TEPPi, ZnTEPPi and CoTEPPi respectively, one can note that as you titrate, the absorbance tends to increase and the Soret bands shift to a longer
wavelength, except for CoTEPPi where the absorption tends to increase without any shifting of the Soret band to either lower or higher wavelengths. This could be due to electrostatic interactions between the DNA phosphate groups and the charged centers or the porphyrazines.

In examining the interaction between TEPPi, ZnTEPPi and CoTEPPi with d(G2T2G2TGTG2T2G2), Figures 45, 55 and 65, one can note that the absorption band for TEPPi and ZnTEPPi are broad and that there is no significant shift of the Soret band, as for CoTEPPi the soret band remains sharp and decreases substantially as the DNA becomes saturated with ligand. This same pattern is seen as you change the oligonucleotide to d(T2G2), as shown in Figures 47, 57 and 67, the Soret band broadens and shifts to a higher wavelength for TEPPi and ZnTEPPi, but for CoTEPPi the Soret band remains sharp and does not broaden. Figure 69 shows the % hypochromicity (+) and % hyperchromicity (-) of the Soret band. The results indicate that calf thymus, d(T2G2), d(T2G2T), and d(G2T2G2TGTG2T2G2) show hypochromicity and that d(G2T2G2) shows hyperchromicity indicating outside stacking by the metallated and unmetallated porphyrazines.

The effects of DNA on the change of wavelength and spectral properties of porphyrazines are collected in Table 3. It indicates that d(G2T2G2TGTG2T2G2) does not effect any change in the λmax for TEPPi and CoTEPPi, but ZnTEPPi does show a shift in the λmax. Calf thymus does not effect any change in λmax, when studied against CoTEPPi, but when studied against TEPPi and ZnTEPPi it shifts the soret band significantly. Based on these studies it can be concluded that TEPPi, ZnTEPPi and CoTEPPi bind with DNA oligonucleotides. Competitive dialysis (see Figure 86) studies indicate that the apparent binding Kapp is relatively low, suggesting that the binding is due to electrostatic interactions.
Interaction of TEPPi with d(T,TG): 

![UV/VIS spectra for the titration of TEPPi in the presence of d(T,TG).](image1)

Figure 41. UV/VIS spectra for the titration of TEPPi in the presence of d(T,G).

![UV/VIS spectra of d(T,G) + TEPPi fully bound and TEPPi unbound.](image2)

Figure 42. UV/VIS spectra of d(T,TG) + TEPPi fully bound and TEPPi unbound.

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Interaction of TEPP1 with d(G7T4G4):

Figure 43. UV/VIS spectra for the titration of TEPP1 in the presence of d(G7T4G4).

Figure 44. UV/VIS spectra of d(G7T4G4) + TEPP1 fully bound and TEPP1 unbound.

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Interaction of TEPP with d(G₂T₃G₂TGT G₂T₂G₂):

Figure 45. UV/VIS spectra for the titration of TEPP in the presence of d(G₂T₃G₂TGT G₂T₂G₂).

Figure 46. UV/VIS spectra of d(G₂T₃G₂TGT G₂T₂G₂)·TEPP fully bound and TEPP unbound.

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Interaction of TEPPi with d(T₅G₃T₃):

Figure 47. UV/VIS spectra for the titration of TEPPi in the presence of d(T₅G₃T₃).

Figure 48. UV/VIS spectra of d(T₅G₃T₃) + TEPPi fully bound and TEPPi unbound.

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Interaction of ZnTEPPI with calf thymus:

Figure 49. UV/VIS spectra for the titration of ZnTEPPI in the presence of calf thymus DNA.

Figure 50. UV/VIS spectra of calf thymus DNA + ZnTEPPI fully bound and ZnTEPPI unbound.

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Interaction of ZnTEPPI with d(T₂G₄):

**Figure 51.** UV/VIS spectra for the titration of ZnTEPPI in the presence of d(T₂G₄).

**Figure 52.** UV/VIS spectra of d(T₂G₄) + ZnTEPPI fully bound and ZnTEPPI unbound.

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Interaction of ZnTEPPI with $d(G_iT_iG_3)$:

**Figure 53.** UV/VIS spectra for the titration of ZnTEPPI in the presence of $d(G_iT_iG_3)$.

**Figure 54.** UV/VIS spectra of $d(G_iT_iG_3) +$ ZnTEPPI fully bound and ZnTEPPI unbound.
Interaction of ZnTEPPI with d(G₂T₃G₂TGTG₂T₂G₂):

Figure 55. UV/VIS spectra for the titration of ZnTEPPI in the presence of d(G₂T₃G₂TGTG₂T₂G₂).

Figure 56. UV/VIS spectra of d(G₂T₃G₂TGTG₂T₂G₂) + ZnTEPPI fully bound and ZnTEPPI unbound.

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Interaction of ZnTEPPI with d(T₇G₇T):

Figure 57. UV/VIS spectra for the titration of ZnTEPPI in the presence of d(T₇G₇T).

Figure 58. UV/VIS spectra of d(T₇G₇T) + ZnTEPPI fully bound and ZnTEPPI unbound.
Interaction of CoTEPPI with Calf Thymus DNA:

**Figure 59.** UV/VIS spectra for the titration of CoTEPPI in the presence of calf thymus DNA.

**Figure 60.** UV/VIS spectra of calf thymus DNA + CoTEPPI fully bound and CoTEPPI unbound.
Interaction of CoTEPPI with $d(T_G)_3$: 

Figure 61. UV/VIS spectra for the titration of CoTEPPI in the presence of $d(T_G)_3$.

Figure 62. UV/VIS spectra of $d(T_G)_3$ + CoTEPPI fully bound and CoTEPPI unbound.

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Interaction of CoTEPPI with d(GT₃G₄):

Figure 63. UV/VIS spectra for the titration of CoTEPPI in the presence of d(GT₃G₄).

Figure 64. UV/VIS spectra of d(GT₃G₄) + CoTEPPI fully bound and CoTEPPI unbound.
Interaction of CoTEPPI with d(G₃T₃G₂TGTG₂T₂G₂):

![UV/VIS spectra for the titration of CoTEPPI in the presence of d(G₃T₃G₂TGTG₂T₂G₂).](image)

**Figure 65.** UV/VIS spectra for the titration of CoTEPPI in the presence of d(G₃T₃G₂TGTG₂T₂G₂).

![UV/VIS spectra of d(G₃T₃G₂TGTG₂T₂G₂) + CoTEPPI fully bound and CoTEPPI unbound.](image)

**Figure 66.** UV/VIS spectra of d(G₃T₃G₂TGTG₂T₂G₂) + CoTEPPI fully bound and CoTEPPI unbound.
Interaction of CoTEPPI with d(T₄G₃T):

**Figure 67.** UV/VIS spectra for the titration of CoTEPPI in the presence of d(T₄G₃T).

**Figure 68.** UV/VIS spectra of d(T₄G₃T) + CoTEPPI fully bound and CoTEPPI unbound.
Figure 69. % Hypochromicity (+) and % Hyperchromicity (-) of the Soret band for TEPPi, MnTEPPi, CoTEPPi, CuTEPPi and ZnTEPPi in the presence of various DNAs.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>TEPPi ΔA</th>
<th>CoTEPPi ΔA</th>
<th>ZnTEPPi ΔA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caif thymus</td>
<td>21</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>d(TaG4)</td>
<td>32</td>
<td>54</td>
<td>23</td>
</tr>
<tr>
<td>d(TaG4T)</td>
<td>35</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>d(GaTeG4)</td>
<td>-32</td>
<td>-1</td>
<td>-25</td>
</tr>
<tr>
<td>d(G2T2G2TGTG2T2G2)</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 3: Effects on DNA on the change of (ΔA) spectral properties of porphyrines
AFM Results:

AFM images of CuTEPPI were measured at 10 μM, 20 μM, 30 μM, 60 μM, 100 μM and 300 μM concentration. It was determined that at these concentrations the length of linearized plasmid prS316 did not change as indicated by Figures 70 – 76. Also, AFM images of CoTEPPI at 10 μM, 30 μM, and 524.5 μM in the presence of linearized plasmid prS316 did not change the length of DNA as shown in Figures 77 – 79. It was also shown that AFM images of MnTEPPI, ZnTEPPI and TEPPI at 300 μM concentration did not change the length of linearized plasmid prS316 (See Figures 80 – 82). Based on our findings we determined that porphyrazines, either nonmetallated or metallated, do not increase the length of linearized plasmid prS316 which contains 4887 base pairs (see Figure 83). Initial studies comparing the DNA lengthening with CoTEPPI and CuTEPPI to the ethidium bromide theoretical curve indicated that there was no change in the DNA length as shown in figure 84.

In order to see if increasing the concentration of porphyrazine from 10 μM to 500 μM would affect the length of the DNA, we found that increasing the concentration of the porphyrin does not change the length of the linearized plasmid DNA. What one can note that when increasing the concentration of porphyrazine, nodes form at different length intervals within the DNA strand. Competitive binding studies done by Bottomley indicated that porphyrazines TEPPi, ZnTEPPI and CoTEPPI, in the presence of ethidium bromide, a well known intercalator of linearized plasmid prS316 displaced the ethidium bromide after reaching equilibrium. Our future work will be to develop a DNA strand in which we can form quadruplexes at certain length intervals within the DNA strands.
Figure 70. AFM image of control linearized plasmid prS316.

Figure 71. AFM of CuTEPPI at 10µM in the presence of linearized plasmid prS316.
Figure 72. AFM of CuTEPPI at 20 \( \mu \text{M} \) in the presence of linearized plasmid pRS316.

Figure 73. AFM of CuTEPPI at 30 \( \mu \text{M} \) in the presence of linearized plasmid pRS316.
Figure 74. AFM of CuTEPPI at 60 μM in the presence of linearized plasmid prS316.

Figure 75. AFM of CuTEPPI at 100 μM in the presence of linearized Plasmid prS316.
Figure 78. AFM of CoTEPPI at 30 μM in the presence of linearized plasmid prS316.

Figure 79. AFM of CoTEPPI at 524.5 μM in the presence of linearized plasmid prS316.
Figure 80. AFM of MnTEPPi at 300 µM in the presence of linearized  
*plasmid prR316.*

Figure 81. AFM of ZnTEPPi at 300 µM in the presence of linearized  
plasmid prS316.
Figure 82. AFM of CoTEPPI at 300 µM in the presence of linearized plasmid µS316.
Figure 83. % DNA strands (%) versus DNA length (μm) from porphyrazine study using AFM (Bottomley, 2004).
Figure 84. Comparison of DNA lengthening from CoTEPPI and CuTEPPI to the ethidium bromide binding curve (Bottomley, 2004).
Competitive Dialysis studies:

Our results, as shown by Figure 83, indicate that porphyrazines do not bind to single stranded poly (dA) or poly (dT) DNA. Figure 84 indicates that we have also found that double stranded poly (dG-dC) and poly (dA-dT) DNA have a low binding affinity ($K_{off}$) to Mn$^{2+}$TEPP, Co$^{2+}$TEPP, Cu$^{2+}$TEPP, Zn$^{2+}$TEPP and TEPPI. We have also found that Mn$^{2+}$TEPP and Zn$^{2+}$TEPP show a greater binding affinity relative to calf thymus DNA (Figure 85) when comparing the difference in the amount of bound relative to the amount bound to calf thymus DNA $C_b - C_b^{DNA}$. On the contrary, the data in Figure 86 indicate that Mn$^{2+}$TEPP, Co$^{2+}$TEPP, Cu$^{2+}$TEPP, Zn$^{2+}$TEPP and TEPPI have a greater affinity for quadruplex structures as indicated by the difference in the amount of bound relative to the amount bound to G$_4$T$_4$ ($C_b - C_b^{T_4}$).
Figure 85. Competitive dialysis diagram showing bound (uM) porphyrine to oligonucleotide DNA.

Figure 86. Apparent binding constant $K_{app}$ diagram of porphyrine for each oligonucleotide of DNA.

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Figure 87. \( C_h - C_p \) = The difference in the amount bound (\( \mu M \)) relative to the amount bound (\( \mu M \)) to calf thymus DNA.

Figure 88. \( C_h - C_p \) = The difference in the amount bound (\( \mu M \)) relative to the amount bound (\( \mu M \)) to d(T,G).
Utility of Porphyrinones as HIV inhibitors:

Reverse transcriptase (RT) plays a key role in the replication of HIV by converting single-stranded genomic RNA into double-stranded proviral DNA and represents one of the main targets for the development of AIDS therapy. Most inhibitors of RT described in the past years, whether nucleoside analogues or non-nucleoside inhibitors target the polymerase activity of RT but present some limitations including toxicity and the emergence of resistant strains of HIV.

The FDA has approved 16 drugs for treatment of HIV infection, and ten of these target the DNA polymerase activity of reverse transcriptase. The downfall is that multi drug resistant HIV strains are becoming increasingly common. Many of these strains are also resistant to most other drugs that are being developed, since these are directed at the same target as the approved drugs. Thus, identification of novel compounds that inhibit HIV targets is essential. In this context, HIV RT-associated RNase H has not been fully researched. Only a few inhibitors of HIV RNase H have so far identified and of those most have poor potency, lack "drug-like" properties, or lack antiviral activity. Only two are readily amenable to medicinal chemistry, the N-acyl hydrazones and the aryl hydrazones, and both classes of inhibitors have problems that may preclude further development. A major barrier to the discovery of RNase H inhibitors is the lack of a suitable assay. Commonly used assays are cumbersome, time-consuming and unsuitable for high-throughput screening (HTS) of large chemical libraries. Parniak has developed a fluorescence resonance energy transfer (FRET) assay that allows high-throughput robotics screening for inhibitors of HIV RNase H. This assay was designed to measure non-specific RNA cleavage reactions in order to be useful in secondary screens with human RNase H. The assay is suitable for both 96-well and 384-well
microplate formats. His group has screened several different chemical libraries with the FRET RNase H assay and shown that the assay is robust, precise (coefficient of variation about 6%, Z-factor of 0.8), provides an excellent "hit rate" (about 0.5%), and yields data comparable to that obtained in standard gel assays. These screens have identified several new structural classes of RNase H inhibitors including those based on the mappicine pharmacophore, a structure highly amenable to combinatorial synthetic approaches that allow focused structural modifications designed to improve inhibitory potency. The FRET assay has also facilitated development of our N-acyl hydrazone RNase H inhibitors, especially reduced-toxicity analogs that will be useful in resistance studies. Parniak's group screened our porphyrines using this high-throughput screening FRET assay for RNase H activity.

The results are summarized in Figure 89. Based on this assay, at a 10 μM concentration of porphyrine, it was determined that TEPPI and ZnTEPPI are relatively potent inhibitors of this HIV activity at 92% and 83% inhibition, respectively. The other porphyrines, MnTEPPI (17%), CuTEPPI (25%), CoTEPPI (12%) did not show high inhibition of HIV reverse transcriptase-associated ribonuclease H activity. These same porphyrines were also tested at 6μM concentration. The results are also shown in Figure 89. At this concentration it was found that TEPPI had a 98% inhibition of HIV activity, followed by TEPPCI at 80% inhibition, followed by MgTEPPI having a 65% inhibition of HIV activity. Cytotoxicity was measured in cell cultures by Dr. Parniak's laboratory. The values to compare are the antiviral data (Figure 90) with the cytotoxicity data (Figure 91) as indicated by Figure 92. The ratio of CC50/EC50 gives the therapeutic index: the higher this ratio, the better the candidate for targeting the virus. What can be summarized about porphyrines is that they are potent RT inhibitors. They have also shown to inhibit the isolated RNase domain fragment. One of the drawbacks is the high cytotoxicity, hence they could be used as microbicides. The solubility
in water and the above findings will help in designing new and less toxic molecules with high inhibition activity.

Figure 89. Intact Reverse transcriptase Rnase H % inhibition at 6 μM and 10 μM concentration. (Parniak 2004).

Figure 90. Concentration of Porphyrine required for 50% inhibition of viral replication of HIV infected cells. (Parniak 2004)
Figure 91. Concentration of porphyrine required for 50% cell kill of the uninfected cells (Parmak 2004).

Figure 92. CC50/EC50 measure of the porphyrine therapeutic index (Parmak 2004).
CHAPTER V
CONCLUSION

The sole purpose of our study was to synthesize new novel nonmetallated and metallated quaternized porphyrines and assess their utility as potential drugs for the treatment of cancer and AIDS. First, we were able to synthesize new novel porphyrines using inexpensive materials which required no elaborate process equipment, while obtaining good yields and high purity compounds. The reason why we obtained one isomer of tetrapyridino porphyrin (TPP) is supported by the fact that the carbon of the nitrile at the para position of 3,4-pyridine-dicarbonitrile has different reactivity than the carbon of the nitrile at the meta position due to resonance effect. This in turn makes the carbon of the nitrile at the para position more positively charged and susceptible to nucleophilic attack. This difference in reactivity obtained results in obtaining one isomer in the tetramerization process which is also supported by the UV/visible spectra obtained in Figure 37. Secondly, we were able to demonstrate through UV/visible studies, atomic force microscopy, and competitive dialysis that these molecules interact with DNA oligonucleotides. Finally, Dr. Michael Parniak, at the Division of Infectious Diseases (University of Pittsburgh, Pittsburgh, PA) used a fluorescence resonance energy transfer assay that allowed high throughput robotic screening of HIV Rsase H. The assay was designed to measure non-specific RNA cleavage reactions rather than the specific cleavage reactions of polypurine tract generation. We were able to demonstrate that porphyrinines inhibit reverse transcriptase Rsase H in the aids virus.

Recently, cationic porphyrins have been shown to demonstrate qualities that make these compounds useful as photosensitizers for a large number of biological systems. Cationic
porphyrins are able to interact with DNA bases, inducing DNA lesions upon photoactivation.231
Also, the combination of hydrophobic and hydrophilic substituents in the porphyrin structure results in an intramolecular polarity axis, which can facilitate membrane penetration. This combination produced a better accumulation in subcellular compartments and enhanced the effective photosensitization.232
The porphyrinānes that we synthesized in our laboratory have shown to exhibit high absorbances at higher wavelengths in the range of 600 – 700 nm, making them viable candidates as photodynamic therapeutic agents within the photodynamic window for the treatment of tumor cells. Another property that makes these molecules good candidates as photodynamic therapeutic agents is that they are able to absorb even at 5 × 10⁴ molar concentrations and that they are water soluble. One of the goals of our group is to continue to synthesize porphyrinānes that offer better photophysical and biological properties.

The synthesis of these new porphyrinānes has also shown they are viable vehicles for the inhibition of reverse transcriptase from the HIV virus as demonstrated by Figure 89. Even at a very low concentration of 6 μM, porphyrinānes have inhibited reverse transcriptase Rnase H. Porphyrinānes required low concentrations, 12 μM to 2 μM, to inhibit 50% of viral replication of HIV infected cells as depicted in Figure 90.

What was found to be promising is that porphyrinānes require a higher concentration to kill 50% uninfected cells as shown in Figure 91. When examining the therapeutic index, which is the ratio of CC50/EC50, one noted that MnTEPPi has the highest therapeutic index as depicted from Figure 92. What can be concluded about porphyrinānes as potential drugs, is that future work must focus itself upon synthesizing porphyrinānes with functional groups that offer
high inhibition at a low concentration, making the therapeutic index higher. Another future area
of interest will be to synthesize porphyrazines with other functional groups such as sugars and
steroids while at the same time making them quaternized, to offer a better vehicle through the
cell membrane. Another possible area for these porphyrazines could be utilized in inks or as
sensitizers for magnetic media. As research continues in the field of phthalocyanines, chemo-,
regio-, and even stereoselective synthetic methods will be developed as suitably functionalized
systems. Chiral analogues of many phthalocyanine derivatives can be expected to be synthetic
targets in the near future. The greater chemical flexibility of related porphyrazines will also help
facilitate the selective synthesis of low-symmetry single compounds.
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