CHAPTER 2  MATERI AL S AND METHOD

The first section of this chapter discusses in detail about the preparation of chemicals, buffers, concentration determination and DNA annealing process for the study of the drug-DNA binding.

The second section, thereafter, consists of the details of various equipments and their principles used for the analysis of the drug-DNA binding studies. The third section includes the details of NMR experimentation for obtaining the structural information of DNA Oligomers. The fourth section deals with the structural modification of Vincamine and its binding affinity with reference to DNA duplex.

2.1 Chemicals and DNA Sequences

Paclitaxel (Taxol), Vinblastine sulfate, Berberine chloride and were purchased from Sigma-Aldrich Chemicals Pvt. Ltd. New Delhi and Vincristine sulphate were procured from Hysel India Pvt. Ltd. Vincamine was purchased from TCL Chemicals (India) Pvt. Ltd. Chennai. These compounds were used without further purification. Buffer salts, sodium chloride (NaCl), Sodium dihydrogen phosphate (NaH$_2$PO$_4$), disodium hydrogen phosphate (Na$_2$HPO$_4$) and ethylenediamine tetra acetic acid (EDTA) were all of analytical grade. The proposed alkaloids were selected on the basis of Lipinski rule. Except Berberine, all these compounds have indole ring in their structure (Lipinski, 2004).

The list of proposed alkaloids (Figure 2.1) includes;

1. Paclitaxel (Taxol)
2. Vincristine
3. Vinblastine
4. Berberine
5. Vincamine

Calf Thymus (CT) - DNA and other five self-complimentary palindromic DNA decamer and dodecamer sequences (DNA-1 to DNA-5) were procured from Sigma-Aldrich Chemicals Pvt. Ltd., New Delhi and stored at 4 °C.
The proposed control DNA oligomer sequences are

1. DNA 1: 5’-d(GATGGCCATC)_2
2. DNA 2: 5’-d(GATCCGGATC)_2
3. DNA 3: 5’-d(GGCAATTGCC)_2
4. DNA 4: 5’-d(GGCTTAAGCC)_2
5. DNA 5: 5’-d(CGCGAATTCGCG) \_2 (Dickerson Dodecamer)

The DNA oligomer sequences proposed for carrying out our research work were self-complementary and having sequence specific central core. Thus by annealing, they formed a double helix with the help of H-bonds. As the first step, the DNA solutions were prepared in the phosphate buffer at physiological pH of ~7.4. The concentration of DNA oligomers were determined spectrophotometrically using the molar extinction coefficients (Table 2.1). The concentration of alkaloids (drug) was calculated volumetrically.
All experiments were carried out at physiological pH. Since alkaloids contain a structure composed of aromatic rings, the presence of delocalized π-electrons in the alkaloids structure enabling auto-fluorescence phenomena. All the proposed compounds obeyed Beers Law in the concentration range of 10 µM to 25 µM employed in the study. All the Fluorimetric titration experiments were performed in triple distilled deionized water while NMR experiments were performed in Deuterium oxide (D₂O) procured from Sigma-Aldrich Chemicals Pvt. Ltd. New Delhi.

Table 2.1: Five DNA oligomers sequence selected for this study (Oligo Evaluator™, 2013)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>% GC content</th>
<th>Molar extinction coefficient ε</th>
<th>Wavelength (nm)</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-1</td>
<td>5′-d(GATGGCCATC)₂</td>
<td>60%</td>
<td>95000</td>
<td>260</td>
<td>30.2</td>
</tr>
<tr>
<td>DNA-2</td>
<td>5′-d(GATCCGGATC)₂</td>
<td>60%</td>
<td>96600</td>
<td>260</td>
<td>30.2</td>
</tr>
<tr>
<td>DNA-3</td>
<td>5′-d(GGCAATTGCC)₂</td>
<td>60%</td>
<td>92600</td>
<td>260</td>
<td>49.1</td>
</tr>
<tr>
<td>DNA-4</td>
<td>5′-d(GGCTTAGCC)₂</td>
<td>60%</td>
<td>93200</td>
<td>260</td>
<td>49.1</td>
</tr>
<tr>
<td>DNA-5</td>
<td>5′-d(CGCGAATTCCG)₂</td>
<td>66.7%</td>
<td>110700</td>
<td>260</td>
<td>64.9</td>
</tr>
</tbody>
</table>

2.2 Sample Preparation for UV and Fluorescence Titrations

2.2.1 Buffer

Sodium phosphate buffer of pH 7.4 was prepared using mono-sodium dihydrogen phosphate (NaH₂PO₄), di-sodium hydrogen phosphate (Na₂HPO₄), ethylene diamine tetra acetic acid (EDTA) and NaCl. Total [Na⁺] concentration was kept at 20 mM. This buffer was prepared in triple distilled de-ionized water. After the preparation of buffer, it was properly filtered through 0.45 µM Millipore (Millipore, Bangalore) filter and degassed to make a homogenous solution. The pH measurements were performed on a Cyberscan 2100 high precision bench pH meter with an accuracy of > ± 0.001 (Eutech Instruments Pvt. Ltd., Singapore). The pH meter was calibrated accurately using the solutions of standard buffer of pH 4.0, 7.0 and 9.2.
2.2.2 DNA Solutions

CT-DNA solution was prepared for fluorimetric titrations. CT-DNA was dissolved in 20 mM phosphate buffer and stored at 4 °C. Purity of the DNA was checked by absorption ratio $A_{260/280}$ in range 1.8-1.9, which indicates that DNA was sufficiently free from protein (Glasel, 1995). The solution was then allowed to homogenize for 2-3 days before utilizing for titrations. DNA oligomer sequences used in fluorescence titrations were dissolved in buffer prepared in water and annealed before titration.

2.2.3 DNA Decamer Annealing Process

The flow chart below shows the procedure adopted in the annealing process (Figure 2.2). Annealing is carried out in order to obtain DNA oligomers in duplex form. Before utilizing the DNA oligomers in fluorescence titrations, all the five DNA sequences DNA-1 to DNA-5 were subjected to annealing process in the presence of buffer solution. Buffer was added in order to stabilize the DNA duplex with the help of Na$^+$ ions and EDTA. Na$^+$ ions are used to counter balance the negative charge on the DNA backbone while EDTA is used to bind selectively with heavy metal ions which might be present as impurity in the DNA solution. For the purpose of NMR experiments, DNA oligomers were annealed in D$_2$O in place of water.

![Flow chart showing the method of DNA annealing](image)

**Figure 2.2: Flow chart showing the method of DNA annealing**

There are several analytical and computational techniques available to study the drug-DNA interactions, for example UV absorption spectroscopy, Fluorescence spectroscopy, Circular Dichroism spectroscopy, NMR spectroscopy, X-ray crystallography, Molecular modeling, etc. Out of these techniques Fluorescence
spectroscopy, Electrochemistry, Agarose Gel Electrophoresis and Molecular modeling methods were utilized in this study. Initially, all drug compounds (1-5) were titrated with CT-DNA using Fluorescence spectroscopy. However, since most of the drug compounds used in this study absorbed the UV light in the region where DNA also absorbs, it was found out that UV absorbance method was not suitable for the drug-DNA binding studies. The UV-absorbance spectroscopy was therefore used only to evaluate the absorption maxima of alkaloids and DNA oligomers.

2.3 Analytical Techniques for Drug-DNA Interaction Studies

2.3.1 Fluorescence Spectroscopy

Fluorescence spectroscopy is probably one of the techniques used to study interactions between small ligand molecules and DNA duplex. The advantages of molecular fluorescence over the other techniques are its high sensitivity and large concentration range. The most intense and the most useful fluorescence is found in compounds containing aromatic functional groups with low energy π-π transition levels (Jaumot and Gargallo, 2012). Complexation between a ligand molecule and a nucleic acid leads to optical changes that can be used to monitor the binding process. As these drug-DNA interactions frequently involve a reversible mechanism, a determination of the equilibrium binding constant can provide insight into the nature and strength of the underlying intermolecular events. Monitoring of the drug-DNA interactions using spectroscopic methods relies on the fact that the fluorescence and electronic absorption spectra of the free ligands are altered upon binding (Dougherty, 1984). A transition between two different electronic energy levels can be induced by the energy supplied by photons and the electron will ‘jump’ from the relatively low energy group singlet state (S₀) to high energy level excited singlet state (Sₙ).

This emission of light from an upper singlet state to the ground state is known as fluorescence. Upon excitation into higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore [Q] in the lowest vibrational level of S₁. This energy transfer occurs with a rate constant Kq (collisional quenching). This relaxation occurs in about 10⁻¹² s. This collisional quenching is illustrated on the modified Jablonski diagram in Figure 2.3 (Lakowicz, 2006).
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Figure 2.3: A Jablonski (electronic transitions) diagram showing the different transitions processes between the excited states

All the selected anticancer alkaloids viz. Paclitaxel, Vinblastine and Vincristine were found to possess the fluorescence emission characteristics upon excitation with the UV light. Consequently all the compounds were investigated using this method. Fluorescence spectra were obtained on Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA) equipped with xenon flash lamp using quartz cells of 1 cm path length, which was attached with Peltier temperature controller along with Pentium-4 IBM Computer. Figure 2.4 shows the scheme for fluorescence instrumentation employed in the study. A fixed concentration of drug (ligand) solution was titrated with increasing concentration of DNA (receptor). Measurements were made in fluorescence free quartz cell of 1 cm path length.
Figure 2.4: Schematic representation of a fluorescence spectrophotometer. The excitation and emission monochromators have variable band pass filters

Emission spectra of free and bound alkaloid were measured according to the reported method by Le Pecq and Paoletti, 1967. For all spectrofluorimetric studies spectral plotting parameters were adjusted according to the fluorescence of the compounds. The excitation band pass was fixed at 5 nm while different emission band pass wavelengths were used (5 nm and 10 nm). The scan speed of 240 nm/min was kept fixed during all the experiments. To study the interaction of anticancer alkaloids with the B-form DNAs, all the experiments were carried out at 25 ºC. Initially, the compounds were titrated with CT-DNA and the effect on the fluorescence peak was noted after each addition of DNA aliquot. Later these compounds were titrated with five DNA decamer sequences (DNA-1 to DNA-5).

2.3.1.1 Analysis of Experimental Binding Data

Double reciprocal method (Benesi and Hildebrand, 1949) was used to calculate the binding constants ($K_a$) of the drug-DNA complexes where the binding affinity was small, with no isosbestic points and Scatchard analysis is not feasible. The Benesi-Hildebrand method of analysis is also employed when oligonucleotide sequences are used instead of large DNA/RNA sequences. In this study, we have used 10 base pairs long self-complementary DNA and therefore this method of Benesi-Hildebrand is employed.
By assuming that there is only one type of interaction between the drug and the DNA in an aqueous solution, equation 1 and 2 can be established;

At equilibrium

\[
\text{Drug} + \text{DNA} \overset{\text{equilibrium}}{\leftrightarrow} \text{Drug-DNA complex}
\]

\[
L + P \overset{\text{equilibrium}}{\leftrightarrow} LP \tag{1}
\]

Here, \(L\) represents the drug (Ligand) concentration and \(D\) represents the DNA concentration.

The equilibrium constant of the Equation [1] will be given as,

\[
K_a = \frac{[LD]}{[L][D]} \tag{2}
\]

Where \([LD]\) is concentration of the complex, \([L]\) is the concentration of free drug and \([D]\) signifies the concentration of the DNA.

Consider the Equation [3] in which the relationship between apparent fluorescence quenching and DNA concentration is given as follows.

\[
\frac{1}{\Delta E_{ap}} = \frac{1}{\Delta E} \frac{1}{K_a[D]} + \frac{1}{\Delta E} \tag{3}
\]

Here \(\Delta E_{ap}\) is the apparent difference in fluorescence and \([D]\) is the DNA concentration.

The plot between \(1/\Delta E\) vs \(1/\text{[DNA]}\) will give a straight line curve with the equation of line defined as.

\[
y = mx + c \tag{4}
\]

The slope \(c/m\) gives the apparent binding constant \(K_a\) value.

### 2.3.2 Electrochemistry

The electrochemical analysis of the drug-DNA interactions is mainly based on the differences in the redox behavior of the nucleic acid-binding molecules in the absence and presence of DNA, including the shifts of the formal potential of the redox couple and the decrease of the peak current resulting from the dramatic drop in the diffusion coefficient after association with DNA (Thevenot et al., 1999).
2.3.2.1 General Procedure

Electrochemical measurements were carried out at AUTOLAB PGSTAT 302N (EcoChemie B.V., Utrecht, The Netherlands) potentiostat-galvanostat with IME 663 and NOVA 1.8 software. All measurements were carried out at room temperature. The voltammetric experiments were performed in a standard three electrode assembly incorporating Glassy Carbon Electrode (GCE) as working electrode (Metrohm India Ltd., diameter = 2 mm), Ag/AgCl (3 M KCl) as reference electrode and platinum wire as counter electrode (Tichoniuk et al., 2010). The surface of working electrode (2 mm diameter) was cleaned before modification. The GCE surface was firstly polished with alumina slurry on polishing pad (using Polishing Kit from BAS Inc., USA), and subsequently cleaned in deionized water obtained from Elga Water Purifier (Shankara et al., 2008). The oxidation of guanine was used as an analytical marker signal and was obtained by using Differential Pulse Voltammetry (DPV).

BR Buffer: Britton-Robinson Buffer is the most widely used buffer media for electrochemical studies, since it provides a wide pH range from acidic (pH 2) to basic (pH 12). BR buffer was prepared by mixing various volumes of acidic buffer and basic buffer. Acidic buffer was prepared by mixing 2.5 gm of $\text{H}_3\text{BO}_3$ [Merck, AR Grade], 2.14 ml of orthophosphoric acid [CDH, AR Grade] and 2.3 ml of acetic acid in 1 Litre of double distilled deionized water. Basic buffer was prepared by dissolving 4 gm of NaOH in 500 ml of water. Solutions of various pH were prepared as shown in Table 2.2.

<table>
<thead>
<tr>
<th>pH</th>
<th>Volume of Acidic Buffer (ml)</th>
<th>Volume of Basic Buffer (ml)</th>
<th>Volume of Water Added (ml)</th>
<th>Total Volume (ml)</th>
</tr>
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<tbody>
<tr>
<td>2.5</td>
<td>100</td>
<td>10</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>3.8</td>
<td>100</td>
<td>20</td>
<td>80</td>
<td>200</td>
</tr>
<tr>
<td>4.5</td>
<td>100</td>
<td>30</td>
<td>70</td>
<td>200</td>
</tr>
<tr>
<td>5.6</td>
<td>100</td>
<td>40</td>
<td>60</td>
<td>200</td>
</tr>
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<td>6.5</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>7.9</td>
<td>100</td>
<td>60</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>8.8</td>
<td>100</td>
<td>70</td>
<td>30</td>
<td>200</td>
</tr>
<tr>
<td>9.5</td>
<td>100</td>
<td>80</td>
<td>20</td>
<td>200</td>
</tr>
</tbody>
</table>
2.3.2.2 Electrode Pretreatment and DNA Immobilization

For DNA biosensor fabrication, DNA has been immobilized over the working surface of glassy carbon electrode. Prior to immobilization, glassy carbon electrode (GCE) has been polished by using an alumina cleaning kit until a mirror-like surface is obtained. Then the electrode is sonicated to remove alumina from the surface of the electrode, thoroughly washed by using deionized water, and dried. After this, DNA of specific concentrations (5 µl of 50 µg/ml) is immobilized on the GCE surface, and then allowed to dry for 30 - 45 min at room temperature (Figure 2.5).

![Flow chart showing DNA immobilization for recording the Differential Pulse voltammogram](image)

**Figure 2.5: Flow chart showing DNA immobilization for recording the Differential Pulse voltammogram**

2.3.3 Agarose Gel Electrophoresis

Electrophoresis refers to the movement of a charged particle in an electrical field. In solution, the phosphates of the DNA are negatively charged therefore DNA fragments can be forced to migrate towards the positive pole through the gel made of agar using an electric field. Because of the large pores size, Agarose gels studies are also suitable for plasmid DNA. Selection of small molecules that bind genomic DNA or other
nucleic acids with high specificity is a central requirement for the drug development. Based on the previous DNA binding study (Michael et al., 1983; Pommier et al., 1991; Wyatt et al., 1997) the goal of this study was to find out any sequence specificity present in the plasmid DNA with drug.

2.3.3.1 Preparation and Purification of Plasmid DNA

The closed circular plasmid, pBluescript SK (+) was amplified and purified from *Escherichia coli* strain JM109 according to mini-prep laboratory protocols (Birnboim and Doly, 1979). Plasmids were isolated using an alkaline lysis procedure, purified in a cesium chloride gradient, and then extensively dialyzed against TE [10 mM Tris (tris (hydroxyl methyl) amino methane), 1 mM EDTA, pH 7.5. Following concentration by ethanol precipitation, the DNA was stored in TE at 4 °C. Complete genetic map of Plasmid (pBluescript SK+) as well as different modes of restriction digestion along with restriction enzymes is shown in Figure 2.6.

![Complete genetic map of Plasmid (pBluescript SK+)](https://www.addgene.org/vector-database/1952/)

Protein was removed by extracting with phenol [Finar Chemicals Ltd., AR Grade] / 0.1 % hydroxyquinoline [Glaxo Chemicals Ltd., AR Grade] (equilibrated with TE pH 8)
and 24:1 chloroform [CDH, AR Grade] / isoamyl alcohol [Merck, AR Grade]. The DNA was then precipitated with NaCl and ethanol, re-suspended in deionized water, and stored at 4 °C (Maniatis et al., 1982).

2.3.3.2 Casting Agarose Gel Slabs

Agarose gel (0.5%) [Sigma-Aldrich chemicals Pvt. Ltd., New Delhi] was prepared by boiling of 1 X TAE buffer [CDH, AR Grade] (40 mM Tris, 20 mM acetic acid and 1 mM ethylene diamine tetra acetic acid) for 2 minutes in microwave. Then the gel was cooled to 60 °C and ethidium bromide [Sigma-Aldrich chemicals Pvt. Ltd., New Delhi] was added (5 µl per 100 ml of the gel). The gel was transferred into the electrophoretic bath [Bio-Rad Laboratories India Pvt. Ltd., Gurgaon] containing TAE buffer. Drug samples prepared with 5 % (v/v) bromophenol blue [CDH, AR Grade] and 3 % (v/v) glycerol [Glaxo Chemical Ltd., AR Grade] was loaded into a gel in 5 µl aliquots (Palecek et al., 1998). The electrophoresis was run at constant voltage of 100 V and 6 °C for 60 minutes. The bands were visualized using a gel projection system at 312 nm (Vilber-Lourmant, France).

2.3.3.3 Restriction Analysis of Plasmid (pBlueScript SK+) by Gel Electrophoresis

5 µg of plasmid DNA (2500 ng / µl) was digested with Restriction Enzymes EcoR-I, Sma-I and Fok-I [Sigma-Aldrich Chemicals Pvt. Ltd., New Delhi] in 20 µl reaction mixture and incubated at 37 °C for 60 minutes. 5 µl Tracking dye bromophenol (4 X) was added before loading on gel. Naturally plasmid exists in different super-coiled forms like covalently closed circles and opens circles. EcoR-I and Sma-I are unique cutter for this plasmid which cut the PBSK at position 701 and 715 respectively and all different forms converts into linear form of 2958 bp. These samples were analyzed on a 0.5 % native agarose gel in TAE buffer containing 0.5 mg/ml ethidium bromide. The gel was visualized and photographed under UV illumination. Restriction enzymes were added at a concentration of 1 µg of DNA in a total reaction volume of 50 µl.

2.3.3.4 Poly Acrylamide Gel Electrophoresis

Non-denaturing Poly Acrylamide Gel Electrophoresis (PAGE) was used for detecting the sequence specific DNA-binding drugs. The various selected DNA primers [Thermo Fisher Scientific, Mumbai] (Table 2.3) were premixed with dye i.e. ethidium bromide
to concentration of about 1 mg/ml. After the 12% of poly acrylamide gel formed, the drug was added (10 µl) to each lane (Lavesa et al., 1993). Electrophoresis was carried out under a constant voltage of 1500 V for 2 hr under 1 X TBE buffer and then fixed the gel in 10% acetic acid / 10% methanol. The gels were then de-stained in 150 ml double distilled water for 8 hr.

Table 2.3: Selected Primers used in PAGE Analysis

<table>
<thead>
<tr>
<th>S. No.</th>
<th>No. of Nucleotides</th>
<th>Sequence of Primers</th>
<th>M. W. (Dalton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>20</td>
<td>5’-GGTAGTCCACGCCTAAACG-3’</td>
<td>6127</td>
</tr>
<tr>
<td>2.</td>
<td>22</td>
<td>5’-GTGCCAGCAGCCGGTGTAATAC-3’</td>
<td>6745</td>
</tr>
<tr>
<td>3.</td>
<td>23</td>
<td>5’-GTGTGACGCGGCGGTGTAACAAG-3’</td>
<td>7201</td>
</tr>
<tr>
<td>4.</td>
<td>25</td>
<td>5’-GATTACTAGCAGCTACCTCATG-3’</td>
<td>7632</td>
</tr>
<tr>
<td>5.</td>
<td>27</td>
<td>5’ACCCACACCAGCCTGCAGGCCGGTGAA-3’</td>
<td>8224</td>
</tr>
<tr>
<td>6.</td>
<td>28</td>
<td>5’-CAGGTGCTGTGGGTTGGGTGAAGATG-3’</td>
<td>8836</td>
</tr>
<tr>
<td>7.</td>
<td>33</td>
<td>5’-GAGGAAGCTTCACACAGCCGGGAAATGGGAGAAGG-3’</td>
<td>10319</td>
</tr>
<tr>
<td>8.</td>
<td>34</td>
<td>5’-TGGTACCATGAGGCCCCCGATGTCTGCTGAC-3’</td>
<td>10396</td>
</tr>
<tr>
<td>9.</td>
<td>39</td>
<td>5’-GTCGACCAGGGATTCCAATGAGGAGCGCTTTATTACTATC-3’</td>
<td>11933</td>
</tr>
<tr>
<td>10.</td>
<td>40</td>
<td>5’-AATAGGATCCTTTGCAATTAATGGCTTTGTCTTTGAT-3’</td>
<td>12336</td>
</tr>
</tbody>
</table>
2.3.4 Theoretical Studies: Molecular Modeling

Molecular modeling is a generic term that refers to computational techniques which are used to depict, describe, or evaluate any aspect of the properties or structure of a molecule (Pensak, 1989). The technique is widely used for studying not only small chemical molecules but also large biological systems. The basic principle of molecular modeling is to assign atomic positions in Cartesian space or in an internal coordinates. It enables three-dimensional visualization and manipulation of the molecular systems in the atomistic level whilst wet experimental studies are certainly difficult to provide information of such a level. Molecular properties (e.g. molecular energy, enthalpy, and binding energies) and its behavior in the presence of other molecules (e.g. electrostatic potentials) are able to be predicted by performing a range of calculations.

Docking procedures were employed to build a suitable model for drug-DNA binding with small molecules and to analyze the resultant orientation of the drug-DNA complexes in the light of the experimental results. Before docking, all the compounds were energy minimized to eliminate bad geometries and steric clashes. The docking calculations were accomplished using online docking server. The structural coordinate files of Paclitaxel, Vinblastine, Vincristine and Vincamine and its derivatives were generated in PDB (protein data bank) format using Discovery Studio program from Accelrys. For DNA docking experiments, the structures (pdb files) were submitted online along with the DNA sequence to the SCFBIO (supercomputing facility for bioinformatics and computational biology) server at IIT-Delhi for docking calculations.

The flow chart depicting the docking method (Figure 2.7) included 4 steps (Gupta et. al., 2007)

a. Identification of the best possible grid/ translational points in radius of 3 Å around the reference point (centre of mass);

b. Generation of grid and preparation of energy grid in and around the active site of the DNA to pre-calculate the energy of each atom in the candidate ligand;

c. Monte Carlo docking and intensive configurational search of the ligand inside the active site; and
d. Identification of the best docked structures on an energy criterion and prediction of the binding free energy of the complex.

![Docking methodology diagram](modified from Pandya, 2009)

The selected docked complexes were energy minimized in vacuum by using AMBER (Pearlman, et al., 1995) force field. For vacuum minimizations, 1000 steps of steepest descent and 1500 steps of conjugate gradient were carried out. This resulted in some conformational corrections in the DNA duplex. The methodology was initially devised for proteins and was later found suitable for the DNA - ligand complexes as well.
The docked structures obtained from the DNADOCK program were later subjected to theoretical energy calculations using PreDDICTA program at IIT-Delhi. The PreDDICTA (Shaikh and Jayaram, 2007) server performs energy calculations according to the following energy function.

\[ \Delta G^0_{\text{cbe}} = \Delta H^0_{\text{el}} + \Delta H^0_{\text{vdw}} - T\Delta S^0_{\text{rt}} + \Delta G^0_{\text{w}} \]  

Here, \(\Delta G^0_{\text{cbe}}\) denotes total binding free energy, \(\Delta H^0_{\text{el}}\) denotes electrostatic energy term, \(\Delta H^0_{\text{vdw}}\) denotes van der Waal’s energy term. \(T\Delta S^0_{\text{rt}}\) signifies the rotational and translational entropy changes on complex formation while \(\Delta G^0_{\text{w}}\) stands for the energy term associated with reordering of waters around DNA and ligand upon binding and is calculated in term of hydration free energy. The energy minimized docked structures of the drug-DNA complexes were generated and analyzed for their binding energies and other structural features.

### 2.3.4.1 Analysis of Molecular Modeling Data

Various sets of binding free energy \(\Delta G\), binding constant \(K_a\) values from experimental and theoretical techniques were secured. The DNA binding constants were obtained from the fluorescence titrations henceforth termed as \(K_{\text{exp}}\) which were used to calculate the \(\Delta G\) values with the help of the Vant Hoff’s Equation 6, appropriately called as \(\Delta G_{\text{cal}}\).

\[ G = -RT \ln K \]  

Here, \(\Delta G\) defines the binding free energy, \(R\) is Gas constant, \(T\) is temperature in Kelvin and \(K\) is the binding constant. We have used the values of temperature as 300 Kelvin and \(R\) as 1.987 cal·K\(^{-1}\)·mol\(^{-1}\). The binding free energy obtained was then converted to kcal/mol.

The PreDDICTA module gave theoretical \(\Delta G_{\text{PreDD}}\) and other energy term values. These \(\Delta G_{\text{PreDD}}\) values were in turn used to calculate \(K\) values for docked structures, which were defined as \(K_{\text{PreDD}}\). Therefore, 4 sets of values were furnished viz., \(K_{\text{exp}}, \Delta G_{\text{cal}}, \Delta G_{\text{PreDD}}\) and \(K_{\text{PreDD}}\) (Figure 2.8). Molecular docking experiments resulted in the generation of 3D docked structures of the drug-DNA complexes in protein data bank.
format. These docked structures were analyzed in detail for their structural features using Discovery Studio visualizer.

![Diagram](image)

**Figure 2.8:** Diagrammatic representation of different values of $\Delta G$ and $K$ obtained from experimental and theoretical methods

### 2.3.4.2 Purine-Pyrimidine Specificity and Minor Groove Width Calculations

As reported earlier (Kumar, et al., 1980; Neidle, 1997), the minor groove widened at the binding site in a drug-conjugated DNA structure to accommodate the drug molecule in a sequence specific binding in the minor groove of DNA oligomer. The possibility of structural perturbations of the DNA structure due to drug binding was also investigated by analyzing distances between opposite phosphorous atoms of the DNA backbone in Figure 2.9 (Stoffer and Lavery, 1994).

Although, the results obtained from docking experiments did not show any base sequence specificity. They, however, indicated purine-pyrimidine specific patterns. These patterns were obtained by identifying the location of the drug onto the DNA sequence and numbering the nucleotide residues from the 5’-end on strand 1. Figure 2.10 shows DNA nucleotide residues numbered from B 1 to B 10 in strand 1 and from B 11 to B 20 in strand-2. Strand 1 was selected as a reference strand and base pairs
were selected based on the location of the drug molecule from 5’-end of the DNA duplex. For example, if drug is present between base pairs B4 - B7 and their corresponding base pairs in opposite strand are B17 - B14. The purine-pyrimidine pattern was identified and reported for strand 1 (B4 - B7) only and not including B17 - B14, just as base sequence specificity is designated. For example, each drug forms complexes with five DNA sequences; therefore, one set of pattern was assigned for any two complexes which showed best similarity. These patterns were termed as follows: ‘same’ stands for same pattern for purines-pyrimidines specificity; ‘1 base altered’ stands for pattern where only one base is different between the two complexes; ‘2 bases altered’ stands for 2 bases are different and ‘position altered’ stands for difference in the position of the base(s) although the number of purines and pyrimidines remains same on the binding site (Pandya et al., 2010).

Figure 2.9: DNA groove parameter of standard B-DNA: width distances in Angstrom; phosphorus atoms is shown in polyhedron shape (Blue in Color)
Figure 2.10: Diagrammatic representation of DNA where P represents Phosphate group, S denotes Sugar moiety and B represents Base
2.4 Structural Study

2.4.1 Nuclear Magnetic Resonance Spectroscopy

NMR structural biologists are always seeking ways to increase the size limit of biological macromolecules that can be studied using NMR techniques to expand the range of biological questions that can be addressed. NMR has proved to be a valuable tool in the determination of structure and dynamics of biological macromolecules in aqueous solution, under conditions similar to those found in native biological systems.

Macromolecular structure determination using NMR spectroscopy typically involves three steps: Following initial recording of NMR spectra, correlations between atoms and resonance peaks are established by means of spectral assignment.

All proton NMR experiments were obtained at the regional NMR facility at Indian Institute of Technology (IIT), Roorkee on a 500 MHz Bruker AVANCE 500 spectrometer equipped with Siemens workstation with Topspin NMR software. Once processed, the analysis of the NMR spectrum was accomplished on SPARKY software developed at UCSF (Goddard and Kneller, 2006).

2.4.2 Two Dimensional NMR Techniques (2D-NMR)

The concept of 2-dimensional NMR was first proposed by Jeener and coworkers’ (1979). Since that time, a number of experimental techniques have been developed. All two-dimensional experiments contain a variable time delay (t1 delay) that modulates some observed property. Fourier transform of the free induction delays (FIDs) obtained gives the familiar intensity versus frequency plot; however each frequency point was also modulated by the t1 delay. This time domain signal can be subjected to the normal Fourier transform analysis and a plot of intensity at two frequencies obtained. The pulse sequences of two-dimensional experiments consist of three sections (Figure 2.11). Preparation sets up the magnetization transfer, mixing allows transfer to occur, and acquisition causes magnetization to enter the XY plane for detection.
Figure 2.11: Stages of a Two-dimensional experiment. Pulses (90°) are represented by rectangles and acquisition by the arrow head. All experiments consist of the three main stages however the number of pulses in each stage varies

2.4.2.1 Nuclear Overhauser Effect Spectroscopy (2D-NOESY)

The Nuclear Overhauser Effect Spectroscopy (NOESY) experiment (Bax and Davis, 1985) is a two-dimensional experiment for the determination of the Nuclear Overhauser Effect (NOE) between protons, where cross-peaks between protons represent a through-space dipolar coupling interaction. The utility of the NOESY experiment is that it provides pair wise distance information. It is preferable to use longer mixing time NOESY experiments with higher signal-to-noise ratios. This is important because longer mixing times allow proton pairs 4 to 5 Å apart to build up enough NOE intensity to be observed. It is often these longer distances which are crucial in calculating accurate three-dimensional structures. However, with longer mixing times, spin diffusion becomes more prevalent. If a reasonable starting structure can be obtained, it is possible to use algorithms which calculate pair-wise distances from NOE intensities while taking into consideration spin diffusion pathways available in the starting structure (Borgias et al., 1990).

Spin-spin relaxation is the origin of the nuclear Overhauser effect (NOE). The ability of one nucleus to relax another is highly distance dependant, only being effective within approximately a 4.5 Å radius. Since the effect is proportional to $1/r^6$, quantification of the NOE allows an accurate determination of the distance and a means of studying through-space interactions.

With the help of NOESY experiments, the assignment of non-exchangeable protons of DNA can be accomplished. Several regions of the NOESY spectrum furnish the assignment of various non-exchangeable protons (Table 2.4). A typical “NOESY walk” of the DNA sequence can be obtained in the Base – H1’ region of the spectrum, i.e.,
between 5.3 - 6.3 ppm in (ω1) dimension vs 7.1 - 8.4 ppm in (ω2) dimension. This region is called as the fingerprint region of the DNA spectrum.

Table 2.4: $^1$H Chemical shift ranges in DNA duplex NMR spectrum

<table>
<thead>
<tr>
<th>Region</th>
<th>$\delta$ (ppm)</th>
<th>Proton Assignment</th>
</tr>
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<tbody>
<tr>
<td>2’</td>
<td>1.8-3.0</td>
<td>H2’, H2”</td>
</tr>
<tr>
<td>4’, 5’</td>
<td>3.7-4.5</td>
<td>H4’, H5’, H5”</td>
</tr>
<tr>
<td>3’</td>
<td>4.4-5.2</td>
<td>H3’</td>
</tr>
<tr>
<td>1’</td>
<td>5.3-6.3</td>
<td>H1’</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>1.2-1.6</td>
<td>T-CH$_3$</td>
</tr>
<tr>
<td>5</td>
<td>5.3-6.0</td>
<td>C- H5</td>
</tr>
<tr>
<td>6</td>
<td>7.1-7.6</td>
<td>C-H6, T-H6</td>
</tr>
<tr>
<td>2, 8</td>
<td>7.3-8.4</td>
<td>A-H8 and G-H8, A-H2</td>
</tr>
<tr>
<td>-NH$_2^*$</td>
<td>6.6-9.0</td>
<td>-NH$_2$ of A, C and G</td>
</tr>
<tr>
<td>-NH*</td>
<td>12.0-15.0</td>
<td>-NH of T and G</td>
</tr>
</tbody>
</table>

*Observed only in H$_2$O

A fingerprint region of a NOESY NMR spectrum of DNA consists of cross peaks between the DNA base protons (H8 protons of Adenine and Guanine; and H6 protons of Cytidine and Thymine) and H1’ protons of deoxyribose sugars of each residue. These cross peaks are of two types, viz., inter-residue cross-peaks between H1’ proton of one nucleotide with the base proton of the next nucleotide residue and intra-residue cross-peaks between base protons and H1’ protons of sugar of the same residue (Figure 2.12 and 2.13). This region, therefore unambiguously characterizes the complete DNA double helical structure. Table 2.5 and 2.6 enlists the set of cross peaks in a typical NOESY spectrum of DNA. Protons H5’ and H5” cannot be unambiguously assigned due to heavy overlapping of cross-peaks and these peaks are of low intensity. Intra- and
inter-nucleotide correlations were identified for all the sugar and the H6/H8 base protons, due to the short connecting distances in standard B-DNA structure.

The well-resolved low-field imino proton resonances were identified in the 1D $^1$H-spectrum. Watson-Crick imino protons appear in the region of 12.0 –14.5 ppm, and indicate the presence of base pairing, and thus were used to confirm the secondary structure. The spectral region has shown cross-peaks between different imino protons, between imino protons and amino protons, and between imino protons and non-labile adenine 2H. There was no exchange peaks between the imino protons and water i.e. the spectral region near $\omega_1 = 4.8$ ppm is devoid of resonance intensity. The pattern of sequential imino-imino connectivities in the 2D-NOESY spectrum allowed adjacent base-pair patterns to be determined, therefore confirmed the DNA sequence (Boelens et al., 1985). Figure 2.12 and 2.13 explaining inter-residue and intra-residue cross-peaks followed by Table 2.5 & 2.6.

![Figure 2.12: NOE cross peaks in NMR spectra of 2'-deoxyribose sugar residue of each nucleotide of DNA. Blue color indicates cross peaks between H1’ to H2’, H2”; cyan color indicates cross-peaks between H3’ to H2’, H2” & H4’; orange color indicates cross-peaks between H1’ and H3’ and red color indicates cross-peaks between H1’ and H4’](image)
Figure 2.13: Intra-residue and Inter-residue connectivities between various protons. These connectivities are observed as NOE cross-peaks in the NMR spectra of DNA. Blue color indicates inter-residue connectivity while red color indicates intra-residue connectivity.

A = adenosine, G = guanosine, C = cytidine and T = thymidine
### Table 2.5: Intra-residue cross peaks in Purine residues found in a typical 2D-NOESY NMR spectrum of DNA

<table>
<thead>
<tr>
<th>Intra-residue cross-peaks between protons</th>
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<tbody>
<tr>
<td>H8</td>
</tr>
<tr>
<td>H1’</td>
</tr>
<tr>
<td>H2’</td>
</tr>
</tbody>
</table>

### Table 2.6: Intra-residue cross peaks in Pyrimidine residues found in a typical 2D-NOESY NMR spectrum of DNA

<table>
<thead>
<tr>
<th>Intra-residue cross-peaks between protons</th>
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</thead>
<tbody>
<tr>
<td>H6</td>
</tr>
<tr>
<td>H1’</td>
</tr>
<tr>
<td>H2’</td>
</tr>
</tbody>
</table>
2.4.2.2 NMR Data Collection

The general scheme for 2D-NMR spectroscopy is:

*Preparation time:* The sample is excited by one or more pulses. This consists of a delay time or a sequence of pulses separated by fixed time intervals.

*Evolution Period* ($t_1$): The resulting magnetization is allowed to evolve for the first time period, $t_1$. The evolution period is the pulse sequence element that enables frequency labeling in the indirect dimension. Further, one or several radiofrequency pulses may be applied to create coherence.

*Mixing time* ($\tau_m$): During this period magnetization is transferred between spins. Mixing sequences utilize two mechanisms for magnetization transfer through scalar coupling or dipolar interaction (NOE). After the mixing period the signal is recorded as a function of the second time variable, $t_2$. This sequence of events is called a pulse sequence.

*Detection Period:* The signal is recorded during the time $t_2$ at the end of the sequence. Detection is often called direct evolution time. During this time the magnetization is labeled with the chemical shift of the second nucleus. The data is recorded at regularly spaced intervals in both $t_1$ and $t_2$ dimensions.

NOESY experiments on DNA-1, DNA-2, DNA-3 and DNA-4 were conducted using the following experimental parameters: Temperature = 298 K, solvent = D$_2$O, Mixing time = 250 ms, Initial delay = 1.5 seconds, Number of scans = 48, Total number of experiments in first dimension = 256, FID resolution = 2.93 Hz / point, Sweep width = 6009 Hz and Pulse program = noesyphpr.

H$_2$O/NOESY parameters: Water NOESY experiments on all DNA duplex (DNA -1 to DNA -4) were conducted using the following experimental parameters: Temperature = 278 K, solvent = 90% H$_2$O –10% D$_2$O, Mixing time = 200 ms, Initial delay = 1.5 seconds, Number of scans = 48, Total number of experiments in first dimension = 256, FID resolution = 2.93 Hz / point, Sweep width = 6009 Hz and Pulse program = WATERGATE.
2.5 Structural Modifications of Vincamine

Vinca alkaloids represent the most important class of anticancer agents e.g. Vinblastine and Vincristine. Among them Vincamine is claimed to have a selective vaso-regulatory action on cerebral circulation.

Since there is no report concerning the possible effect of Vincamine on chromatin components and DNA. The activity of Vincamine at the receptor level is completely unknown. Our aim in the proposed work is to synthesize amide derivatives of Vincamine with potential biological activity. It was expected that the proposed derivatives would also exhibit DNA binding profile, as shown by Vinblastine and derivatives.

2.5.1 Synthesis of Vincaminic acid (5a)

The amide derivative was synthesized in two steps.

Vincamine (5) was converted into Vincaminic acid (5a) using the following synthesis scheme shown in Figure 2.14. Vincamine was treated with strong acid to prepare Vincaminic acid (5a). 250 mg of Vincamine (5) was mixed with 15 ml of 3M HCl and subjected to hydrolysis by heating in a boiling water bath for 7 hours. The completion of reaction was monitored by thin layer chromatography (TLC) using methanol: ethyl acetate: chloroform (2:1:1, v/v/v) as a solvent system.

Hydrolysis of Vincamine was carried out in acidic condition. In hydrolysis the ester group present in Vincamine (5) was converted into corresponding carboxylic acid (5a).

**Figure 2.14: Synthesis scheme for the preparation of Vincaminic acid**
2.5.2 Synthesis of Dialkylamine Derivative of Vincamine (5)

N, N-dialkylvincaminamide (6) was prepared from Vincaminic acid (5a) using the following synthesis scheme shown in Figure 2.15.

To a suspension of 9.7 g (0.03 mol) of Vincaminic acid (5a) in 400 ml of dry benzene [CDH, AR Grade] was added 3.6 g. (2.5 ml; 0.03 mol) of thionyl chloride (SOCl₂) [Merck, AR Grade] and this mixture was then stirred for 3 hours at ambient temperature. The mixture was stirred for about 30 minutes and corresponding dialkylamine was added to the solution and placed for cooling in an ice bath. The stirring was done for three hours. The mixture was poured into 100 ml of 10% ammonia, the whole mixture is stirred vigorously and the organic layer is decanted, washed with water until neutral, dried over dry sodium sulphate and filtered through activated charcoal.

(5a)  
NN-Dialkylvincaminamide

Reactions were monitored using Glass TLC Plates. Purification of compounds secured was done using silica gel Preparative Thin Layer Chromatography (Prep-TLC). IR spectra were measured with a Shimadzu FT-IR instrument. ¹H-Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker 500-AVANCE Spectrometer.

Figure 2.15: Synthesis scheme for the preparation of NN-Dialkylvincaminamide
2.6 Antimicrobial Activity by a Well Diffusion Assay

2.6.1 Media Preparation and Its Sterilization

Potential mechanisms involved in the inhibition of pathogenic bacterial growth were investigated by a well diffusion assay. This set of experiments was run in order to test if the inhibitory effect of the derivatives of Vincamine was exclusively due to increasing the alkylation chain at amide position. The derivatives of Vincamine were used for evaluating antimicrobial effect on the test phytopathogens including Gram-positive (B. subtilis, B. fusiformis) and Gram-negative bacteria (E. coli) and phytopathogenic fungi i.e., A. niger.

<table>
<thead>
<tr>
<th>Table 2.7: Composition of Media</th>
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</thead>
<tbody>
<tr>
<td>1. Peptones [CDH]</td>
</tr>
<tr>
<td>2. NaCl</td>
</tr>
<tr>
<td>3. Beef Extract</td>
</tr>
<tr>
<td>4. Tripled Distilled Water</td>
</tr>
</tbody>
</table>

2.6.2 Agar Well Diffusion Method

For Agar well diffusion method (Murray et al., 1995) antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungal assay, Sabouraud Dextrose Agar media (SDA; 39 gm/L) were used for developing surface colony growth. All the above ingredients were mixed in one litre distilled water and heated to dissolve all the ingredients. The medium was stabilized in autoclave at 15 pound pressure at 121 °C for 20 minutes.

After pouring the agar gel, the plates were incubated under appropriate conditions at 37 °C for 24–48 hrs and antimicrobial activity recorded as growth-free inhibition zones around the wells. The diameter of the inhibition zone (mm) was measured. Triplicates were maintained in the experiment, for each replicates the average values were recorded. About 10 μl (10 % w/v) of derivative product were added with sterile syringe into the wells and allowed to diffuse at room temperature for 2 hrs and then incubate for 24 hrs for bacterial culture and 48-72 hrs for fungal culture.