CHAPTER 2
PSQ-DNA BIOPHYSICAL INTERACTION STUDIES
2.1 Introduction

DNA is a natural product of paramount importance in understanding the mechanism of genetic processes of cell growth and differentiation, of ageing and senescence. It is also a logical target for chemotherapy. Binding of peptides, small organic and inorganic molecules to DNA can interfere with the numerous processes, including transcription and replication in which DNA participates. Such interference can retard or prevent cell growth or even kill the cell. Extensive chemical and biochemical studies have characterized a variety of molecules that bind to DNA (antiviral, antibiotic, antiprotozoal and antitumor agents). Some of these have clinical applications others are under clinical trial.

Small molecules bind to DNA in various ways. Lerman in 1961 proposed that planar polycyclic aromatic molecules interact with DNA through intercalative mode. A new class of planar tetracyclic condensed quinoline molecule Pyrimidoselenoloquinolines (PSQ) and Pyrimidothienoquinolines (PTQ) were synthesized by successive building up of selenophene / thienophene and pyrimidine rings on quinoline by Dimroth rearrangement. This ring system captured our interest because of their structural relatedness to anticancer alkaloid Ellipticine (widely used potent chemotherapeutic agent used in treatment of various cancers like breast cancer, lymphoma and leukemia etc.)

Reports from our lab, based on studies performed to gain insight into different aspects of the association of PSQ/PTQ with DNA revealed their intercalating ability into DNA. Substitution of sulphur (thienophene ring) with selenium (selenophene ring) did not much alter their binding abilities with DNA, but resultant
biological effect had shown significant variation. Though all these molecules exhibited intercalative mode of interaction the strength of binding and their resultant biological activity varied with the type, length and position of their side chains.

These findings prompted us to explore new PSQ analogues (with different side chains) to study their binding features and to discover the origin of their biological activity. In this chapter binding of three new members of PSQ family 4-amino PSQ, 4-benzylamino PSQ and 8-methyl-4-(3-diethylaminopropylamino)PSQ has been studied employing spectroscopic methods like UV-Vis, Fluorescence, Circular Dichroic and Hydrodynamic methods to understand their interaction process, since biological activities of DNA interacting molecules were admitted to be related to their DNA affinity.
2.2. **Materials and Methods**

Materials and general methodology employed in the present study are as described below:

2.2.1 **Chemicals:**

All chemicals used were of analytical grade and are given below from where they are obtained. All aqueous solutions were prepared from analytical grade salts and doubly distilled water.

**Sigma Chemical Co, USA**

- Ellipticine (5,11-Dimethyl-6H-Pyrido[4,3-b]Carbazole),
- *Clostridium perfringens* DNA (28%GC Type-XII),
- Calf thymus DNA (42%GC Type-I),
- *Micrococcus lysodeikticus* DNA (72%GC Type-XI).

**Merck, India:**

- Dimethyl sulfoxide (DMSO)

**HiMedia Laboratories, India:**

- Ethylene diamine tetra acetic acid (EDTA),
- HCl

**Reagent Preparation**

*Tris-HCl Buffer:* Preparation of tris-HCl buffer (pH 7.0): stock solution of 0.2 M tris-(hydroxymethyl amino methane, 24.2 g in 1L) and 0.2 M HCl. 50 ml of 0.2 M tris was mixed with the 47 ml of 0.2 M HCl and the pH was adjusted to 7.0 and diluted to 200 ml with the double distilled water.
**PSQ intercalates to DNA and induces cancer cell death**

**Test Anticancer Compounds:**

The three anticancer test compounds of the family Pyrimido [4,5:4,5] selenolo(2,3-b)quinoline (PSQ),

A. 4-aminopyrimido[4,5:4,5]selenolo(2,3-b)quinoline (4-amino PSQ),

B. 4-benzylaminopyrimido[4,5:4,5]selenolo(2,3-b)quinoline (4-benzylamino PSQ)

C. 8-methyl-4-(3-diethylaminopropylamino)pyrimido[4,5:4,5]selenolo(2,3-b)quinoline (MDPSQ)

were taken up for the present study (Figure 1.1; Chapter I). These compounds were a generous gift from Dr. S.Y. Ambekar, Department of Chemistry, University of Mysore, Mysore, India.

2.2.2 Preparation of Compounds:

The aqueous stock solutions of APSQ, BAPSQ and MDPSQ were prepared by wetting the compound crystals with DMSO, followed by addition of tris-HCl buffer. Stock drug solutions were stored at -20 °C and used within 2-3 weeks. Working stock solutions of each drug were prepared by dilution of aqueous stock solutions with DNA binding buffer (0.05 M tris-HCl buffer, pH 7.0, 0.05 M NaCl, 0.001 M EDTA).

2.2.3 Purification of DNA:

DNA was purified by standard phenol extraction as described in the literature (Mamaus, 1982). The purity of final DNA preparation was checked by monitoring the absorption spectrum and the ratio of absorbance at 260 and 280 nm. The samples were dissolved in 0.1 M tris-HCl buffer pH 7.0. The DNA concentrations were
determined spectrophotometrically at 260 nm using a molar extinction coefficient with respect to nucleotides as shown in the Table 2.1.

Table 2.1: Molar extinction coefficients of different GC containing DNAs at 260 nm

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>DNA type</th>
<th>GC %</th>
<th>Molar extinction coefficient at 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Clostridium perfringens</em> XII</td>
<td>28</td>
<td>6300 M⁻¹cm⁻¹</td>
</tr>
<tr>
<td>2</td>
<td>Calf thymus I</td>
<td>42</td>
<td>6600 M⁻¹cm⁻¹</td>
</tr>
<tr>
<td>3</td>
<td><em>Micrococcus lysodeikticus</em> XI</td>
<td>72</td>
<td>6900 M⁻¹cm⁻¹</td>
</tr>
</tbody>
</table>

2.2.4 DNA Binding measurements:

2.2.4.1 UV-Visible Spectroscopic Measurements

UV-Visible absorption spectrum was determined in a Perkin-Elmer model 554 (Perkin-Elmer, USA), UV-Vis recording spectrophotometer using 1 cm light path length quartz cuvettes. The parameter of interaction between APSQ/ BAPSQ/ MDPSQ and DNA were determined spectrophotometrically. All compounds examined obeyed Beer's law over the range of concentrations used APSQ (3.4 μM), BAPSQ (2.6 μM) and MDPSQ (2.5 μM), and the molar extinction coefficients were determined at their appropriate λₛₘₐₓ values (Table 2.2). Molar extinction coefficients of compounds bound to DNA were determined at the same wavelength as the molar extinction coefficient measurements of the free compound, but a large molar excess of DNA was present ([DNA nucleotides]/[Compound]>10-175).
Spectrophotometric titrations were performed by serial addition of 100-1000 μL aliquots of DNA solution (1.75-197.4 μM) into a 1 cm path length quartz cell containing 10 μL of corresponding compounds (2.5 – 3.4 μM) in tris-buffer and scanning the UV-Visible spectrum after each addition.

Titrations were stopped when no shift to the lower energy range of the maximum absorbance wavelength in the spectrum was detected between additions. These absorbance values were converted to \( r \) (mole of bound compound/mole of DNA base pairs) and \( C \) (molar concentration of free ligand) using the free and bound molar extinction coefficients for the analyzed compound. Experimental data that were plotted using the method of Scatchard (Scatchard, 1949) (results outside of this range are subject to large systematic errors as a result of experimental errors in molar extinction coefficient determinations (Deranleau, 1969)).

The intrinsic binding constants were determined using experimental spectrophotometric readings from absorbance titration experiments conducted at 342 nm (APSQ), 340 nm (BAPSQ), 332 nm (MDPSQ).

**Table 2.2:** Molar extinction coefficients of APSQ, BAPSQ and MDPSQ at their \( \lambda \) max

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength (nm)</th>
<th>Molar extinction coefficient (M(^{-1}) Cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>APSQ</td>
<td>342 nm</td>
<td>7,200 M(^{-1}) cm(^{-1})</td>
</tr>
<tr>
<td>BAPSQ</td>
<td>340 nm</td>
<td>26,990 M(^{-1}) cm(^{-1})</td>
</tr>
<tr>
<td>MDPSQ</td>
<td>332 nm</td>
<td>17,002 M(^{-1}) cm(^{-1})</td>
</tr>
</tbody>
</table>
For each ligand the apparent association constant(s) $K (M^{-1})$ and number of sites per nucleotide ‘$n$’ were estimated from Scatchard plots using the following equation:

$$\frac{r}{C_r} = K_i \left(1-nr\right) \left(1-nr/[1-(n-1)r]\right)^{n-1} \quad (1).$$

The extended neighbor exclusion model of McGhee and von-Hippel (McGhee and von-Hippel, 1974) was employed using a non linear least-squares fitting procedure to derive the binding parameters analyzed for the compounds.

2.2.4.2 Fluorescence Measurements

Fluorescence spectra were determined in a Hitachi model FL-2000 spectrofluorophotometer. Fluorescence spectra of compounds were determined by exciting 1 ml of solution of 12-42 $\mu$M Pyrimidoselenoloquinolines at 299 nm and varying the emission wavelength in the lower energy region of each spectrum. Quenching of compound’s fluorescence by DNA was measured by addition of micro liter volumes of concentrated DNA solutions to the cuvette. The dilution effect caused by addition of the DNA solution was negligible.

All measurements were carried out at 25 °C in DNA binding buffer (0.05 M tris-HCl buffer, pH 7.0, 0.05 M NaCl, 0.001 M EDTA). A minimum of two determinations of binding parameters were made for each compound-DNA combination. Identical preparations of each DNA were used for all compounds. Each binding determination consisted of a minimum of 10 different DNA concentrations, each of which consisted of duplicate or triplicate samples.
The DNA binding of pyrimidoselenoloquinolines was measured by spectrofluorometry in a manner analogue to previous studies (Duvernay et al., 1979; Pesce et al., 1971). This was done by titrating fixed concentration of APSQ (3.4 µM), BAPSQ (2.6 µM) and MDPSQ (2.5 µM), with increasing concentration of DNA, thereby varying the DNA/drug ratios from (0-100). The DNA/drug ratio 100 was taken as the end point in this titration assay, with the drug considered totally bound. Increasing concentrations of DNA in total of 1000 µl of DNA binding buffer were added to a series of acid-clean glass tubes. The binding reaction was initiated by the addition of 10 µl of 4-amino PSQ/10 µl of 4-benzylamino PSQ/10 µl of MDPSQ working stock solution in DNA binding buffer to each tube, followed immediately by mixing. To eliminate fluorescence due to DNA, parallel control titrations consisting of increasing concentrations of DNA in 1ml of DNA binding buffer were run for each experiment.

Compounds were excited at 299 nm and the fluorescence intensities were monitored at 426, 432 and 465 nm, respectively.

The intrinsic binding constant $K_a$ was determined from equation:

$$C/Δε_a = C/Δε + 1/ΔεK_a$$

Where $C$ is the concentration of DNA determined by the absorbance at 260nm, $Δε_a = [ε_a - Δε_i]$, and $Δε = [Δε_b - Δε_i]$, where $Δε_a$, $Δε_b$ and $Δε_i$ correspond to the apparent extinction coefficient of pyrimidoselenoloquinolines (APSQ, BAPSQ and MDPSQ), the extinction coefficient of the bound and free form of pyrimidoselenoloquinolines respectively. In fluorescence quenching experiments, the data were plotted according to the Stern-Volmer equation:
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\[ \frac{I_0}{I} = 1 + K_q [Q] \] (3).

Where, \( I_0 \) and \( I \) are fluorescence intensities in absence and presence of DNA. \( K_q \) is the Stern-Volmer fluorescence quenching constant, which is a measurement of the efficiency of quenching by quencher, [Q], the concentration of quencher.

The intrinsic binding constants of pyrimidoselenoloquinolines with *Clostridium perfringens* and *Micrococcus lysodeikticus* DNA were also determined by fluorescence titrations. Binding curves were plotted as previously described.

2.2.4.3 Viscosity Measurements

For viscometric experiments, the DNA was sonicated to fragments of approximately 4.5 \( \times \) 10^5 Da as described by Eigner and Doty (Eigner and Doty, 1965). The sonicated DNA sample displayed an \( A_{260}/A_{280} \) ratio of 1.92. This spectral data are consistent with published values of Muller and Crothers (Muller and Crothers, 1968). Solutions of sonicated DNA and the drugs were prepared in tris buffer (50 mM, pH 7.0). These solutions had different molar ratio, 'r' of added compounds to DNA nucleotides. Flow rates were measured to an accuracy of 0.01%. Time readings were recorded in triplicate to 0.01s.

Increase in length of short calf thymus DNA segments was determined by measuring the increase in the intrinsic viscosity of the DNA in the presence of increasing concentrations of APSQ, BAPSQ and MDPSQ. Viscometric measurements were made according to published procedures (Larue *et al.*, 1970; Saucier *et al.*, 1971).
Viscometric measurements were carried out using a semi-micro dilution capillary viscometer at 20 °C (± 0.02) in the tris-HCl buffer: sheared gradient was 500 sec\(^{-1}\). Flow times for the equilibrium solutions were about 50-55 sec while the DNA solutions had 5-100 sec longer flow times; the long efflux times made kinetic energy corrections negligible. The reduced viscosity (\(\eta_{red}\)) of sonicated DNA does not depend on its concentration in the 150-170 \(\mu g/ml\) concentration range. Relative increase in the length of rod like DNA molecules, \(L/L_0\) were obtained through the relationship (Cohen and Eisenberg, 1969),

\[
L/L_0 = \left[ \frac{[\eta]}{\eta_0} \right]^{1/3} \tag{4}
\]

2.2.4.4 Melting Temperature Measurements

Thermal denaturing studies were carried out employing APSQ, BAPSQ and MDPSQ with Calf thymus, *Clostridium perfringens* and *Micrococcus lysodeikticus* DNA in a spectrophotometer fitted with a thermostated cell holder according to the procedure of McCoubrey (McCoubrey et al., 1996). The temperature was increased at a rate of 0.33 °C per minute, allowing the system to stabilize for 15 min before taking the first reading. A signal averaging time of 2 s was used. Systems studied were 500 nM - Calf thymus, *Clostridium perfringens* and *Micrococcus lysodeikticus* DNAs with 0 \(\mu M\) or 15 \(\mu M\) APSQ, BAPSQ and MDPSQ.

The increase in absorbance at 260 nm, corresponding to the transition from ds to ss DNA was used to monitor the thermal denaturation of different GC containing DNAs in the absence and presence of the pyrimidoselenoloquinolines. The melting temperature, \(T_m\) is defined as the midpoint of the transition, and is normally calculated by averaging the minimum and maximum absorbance, and interpolating to
the mid-point. An alternative and more accurate method is to take $T_m$ as the point of inflexion, found from the derivative of the melting curve. The advantage of this method is that it is much easier to identify when more than one transition occurs.

2.2.4.5 Circular Dichroism Measurements

CD is one of the most sensitive techniques for the detection of interactions and changes in nucleic acid conformations. Mode of binding and the orientation of the intercalator in its binding site are the issues addressed employing CD technique.

The CD spectra were obtained with a JASCO spectropolarimeter model J-810 A attached with a MPS-60 microprocessor unit at 5°C. A quartz cell of 1 cm path length was used to obtain spectra from 205-360 nm with a resolution of 0.1 nm. The desired ratios of PSQ to DNA were obtained by adding increments of PSQ to the cell containing a constant amount of the DNA. The CD measurements were performed in a 1 mM tris buffer, pH 7.0. Titrations were performed at 5 °C by incrementally adding aliquots of 5 μL ~ 30 μL aqueous solution of APSQ/ BAPSQ/ MDPSQ to 1.0 ml DNA solutions, contained in 1 cm path length non-fluorescent quartz cell.

Complex formation is a balanced process and unbound PSQ remains present in the solution. Since PSQ is a planar and an achiral chromophore, only those molecules complexed to the asymmetric DNA double helix are able to display induced CD. This may concern drug molecules either externally bound at the proximity of the chiral deoxyribose moieties on DNA or intercalated into DNA base pairs. Each binding mode involves particular surroundings and interactions for the drug molecule, so that the arising CD signals may display varied positions, signs and shapes. Ultraviolet absorbance and CD signals were measured at wavelengths
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corresponding to characteristic maxima. Molar circular dichroism (Δε) was used for the CD spectra. The pyrimidoselenoloquinolines / oligonucleotide ratios ‘r’ were expressed in mol/base.
2.3 Results:

2.3.1 UV-Visible Spectroscopic Studies

Binding of a drug/ligand to DNA produces various changes in the drug's spectral properties. Those changes for 4-aminoPSQ (APSQ), 4-benzylaminoPSQ (BAPSQ) and 8-methyl-4-(3-diethylaminopropylamino) PSQ (MDPSQ) analogues were well recorded. The stepwise addition of small increments of native calf thymus DNA to a solution containing a constant concentration of PSQ analogues in initial excess is shown in Fig 2.1a-c. As shown previously by Parker and Irvin (1952) progressive (hypochromism) depression of the absorption peaks of PSQ analogues and a shift of these peaks toward longer wave lengths (bathochromic shift) occurred as a result of formation of more stable complexes, until a limit representing the spectrum of the completely bound ligand was reached. No further spectral changes developed beyond this point, indicating absence of aggregation (Cohen and Yielding, 1965). The presence of an apparent isosbestic point indicated that the spectra of the limiting systems (i.e., spectra of free and completely bound PSQ analogues) intersect (Cohen and Fischer, 1962) and permitted selection of a single wavelength for the study of complex formation. The absorption peak at 342, 340 and 332 nm respectively were chosen to yield measurements of greatest accuracy and a value for α was calculated for each of the spectral curves at this wavelength. The data obtained in this experiment are presented in Table 2.3. These data yielded a non-linear plot of r/C versus r (Figure 2.2 a-c) suggesting that primary polymer structure (i.e., heterogeneity of binding sites) is the basis for such curvature.
Figure 2.1 A, B and C: Binding curves for APSQ (a) BAPSQ (b) and MDPSQ (c) to calf thymus DNA showing the isosbestic points. Spectrophotometric titrations were performed by serial addition of 100 µl aliquots of a DNA solution (1.75-197.4 µM) into a 10 mm path length quartz cell containing a solution of APSQ 3.4 µM, BAPSQ 2.6 µM and MDPSQ 2.5 µM and scanning the UV-visible spectrum after each addition.
Figure 2.2 A, B and C: Scatchard plots for the binding of APSQ (a), BAPSQ (b) and MDPSQ (c) to calf thymus DNA. Values for binding ratio $r$ and free compound concentration $C$ were determined from data taken from spectrophotometric titrations of the compounds by the DNA.
The interaction of APSQ/ BAPSQ/ MDPSQ with DNA resulted in a strong decrease of absorption intensity (42 - 60%) accompanied by a shift towards higher wavelengths by 4, 2 and 2 nm respectively at 342, 340 and 332 nm peak maxima in the presence of an excess of DNA at a molar ratio equal to 12. APSQ did show two isosbestic points at 206 and 261 nm while BAPSQ did show one isosbestic point at 286 nm and MDPSQ did show three isosbestic points at 229, 245 and 288 nm. Hypochromism was suggested to be result of strong interactions between electronic states of the intercalating chromophore and that of the DNA base pairs. The spectral changes that we have observed (including red shift, hypochromicity and isosbestic points) are consistent with the intercalation of the chromophore into DNA base pairs (Table 2.1). From the spectral changes, non-linear Scatchard binding curves were generated for the interaction of PSQ molecules with DNA using the non-cooperative McGhee-Von Hippel equation (Fig 2.2 a-c). The binding parameters $K_a$ and $n$ are shown in Table 2.3 with $K_a$ being apparent binding constant and $n$ the number of base pairs per bound molecule. APSQ, BAPSQ and MDPSQ molecules has $K_a$ values of $7.5 \times 10^4 \text{M}^{-1}$, $8.6 \times 10^4 \text{M}^{-1}$ and $1.32 \times 10^5 \text{M}^{-1}$ and $n$ values of 0.11, 0.117 and 0.13 respectively, which are essentially of the same order of magnitude as that of the well known intercalators.
Table 2.3: DNA binding properties of 4-aminoPSQ, 4-benzylamino PSQ and 8-methyl-4-(3-diethylamino propylamino) PSQ molecules

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Δλ</th>
<th>H %</th>
<th>( ^{2}K_{s} ) ( (10^{4} \text{ M}^{-1}) )</th>
<th>( h_{n} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>APSQ</td>
<td>4</td>
<td>42</td>
<td>7.5 ± 0.02</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>BAPSQ</td>
<td>2</td>
<td>54</td>
<td>8.6 ± 0.056</td>
<td>0.117 ± 0.02</td>
</tr>
<tr>
<td>MDPSQ</td>
<td>2</td>
<td>60</td>
<td>13.2 ± 0.04</td>
<td>0.13 ± 0.05</td>
</tr>
</tbody>
</table>

\( \Delta \lambda = (\lambda_{\text{bound}} - \lambda_{\text{free}}) \), where \( \lambda_{\text{free}} \) and \( \lambda_{\text{bound}} \) are the wavelengths of maximum absorption for free and DNA bound compounds. \( H = \text{percent hypochromicity} \) \( [%H = (1 - c_{\text{bound}} / e_{\text{free}}) \times 100] \), where \( e_{\text{free}} \) and \( e_{\text{bound}} \) are the extinction coefficients for free and DNA bound compounds.

\( ^{2}K_{s} \) = the affinity constant for DNA \( (\text{M}^{-1}) \). \( h_{n} \) = number of base pairs per bound molecule.

2.3.2 Salt effect on binding

The three analogues of PSQ molecules 4-aminoPSQ, 4-benzylaminoPSQ and 8-methyl-4-(3-diethylaminopropylamino) PSQ carry a positive charge and DNA has a negative phosphate skeleton, we investigate the electrostatic interactions between the intercalator and DNA. In order to test if there was an electrostatic interaction between the analogues of PSQ and DNA, the strong electrolyte, sodium chloride (NaCl), was used. The electrostatic interaction would be weakened by the additional counter ion because of the conversion of the electrostatic atmosphere of DNA periphery. Some reports showed that very high concentration of NaCl would hinder small molecules from binding with DNA (Cao and He, 1998). If there did exist electrostatic interaction between compounds and DNA, the strong electrolyte, sodium chloride (NaCl), was used. The electrostatic interaction would be weakened by the additional counter ion because of the conversion of the electrostatic atmosphere of DNA periphery. Some reports showed that very high concentration of NaCl would hinder small molecules from binding with DNA (Cao and He, 1998). If there did exist electrostatic interaction between compounds and DNA, the following phenomenon should happen by increase in the ionic strength of solutions of drugs and DNA. The absorbance of solutions would increase when NaCl was added, but it would never exceed the absorbencies of the drug solution (Table 2.4a-c). This indicated that electrostatic binding was a part of the interaction of drugs and DNA. But the effect was not as great as expected. However, if the adding order of DNA and NaCl was changed, the intrinsic binding constants were decreased greatly from 2.91 – 2.11 X 10^4 M^{-1} (APSQ), 3.12 – 2.32 X
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$10^4 \text{ M}^{-1}$ (BAPSQ) and $3.28 - 2.59 \times 10^4 \text{ M}^{-1}$ (MDPSQ) when the concentration of NaCl (i.e., ionic strength) increased from 0.05 M to 0.1 M (Table 2.4a-c). Approximately 24, 26 and 30 % decrease was observed in the binding constants respectively when the ionic strength increased.

The ionic strength had significant effect on the binding of PSQ analogues with DNA, and the different adding sequence of DNA and NaCl had different effects on the binding of these compounds with DNA (Table 2.4a-c). If NaCl was added before DNA, there existed a significant salt effect on binding, otherwise there was little salt effect. This indicates that there was competitive interaction between drug and NaCl with DNA. The first added cations assembled near the anionic DNA, and hindered the interaction of other cations added afterwards with DNA. So the probable intercalation process was that these compounds were attracted to the periphery of DNA by electrostatic attraction, and then intercalated in to the stack base pairs. However, the salt effect could not inhibit the binding of PSQ analogues with DNA completely.
Table 2.4a: Influence of ionic strength on the binding of APSQ to calf thymus DNA

<table>
<thead>
<tr>
<th>Ionic strength of NaCl (in M)</th>
<th>Adding DNA after NaCl Binding constant (M⁻¹)</th>
<th>Decrease (%)</th>
<th>Adding DNA before NaCl</th>
<th>A/A₀</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>2.91 x 10⁴</td>
<td>---</td>
<td>1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.075</td>
<td>2.6 x 10⁴</td>
<td>11.2</td>
<td>0.9327</td>
<td>6.87</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>2.11 x 10⁴</td>
<td>24.67</td>
<td>0.8878</td>
<td>9.11</td>
<td></td>
</tr>
</tbody>
</table>

A and A₀ are the absorbance of APSQ with and without DNA at 340 nm.

Table 2.4b: Influence of ionic strength on the binding of BAPSQ to calf thymus DNA

<table>
<thead>
<tr>
<th>Ionic strength of NaCl (in M)</th>
<th>Adding DNA after NaCl Binding constant (M⁻¹)</th>
<th>Decrease (%)</th>
<th>Adding DNA before NaCl</th>
<th>A/A₀</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>3.12 x 10⁴</td>
<td>---</td>
<td>1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.075</td>
<td>2.68 x 10⁴</td>
<td>12.5</td>
<td>0.9121</td>
<td>7.32</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>2.32 x 10⁴</td>
<td>26.12</td>
<td>0.8648</td>
<td>11.41</td>
<td></td>
</tr>
</tbody>
</table>

A and A₀ are the absorbance of BAPSQ with and without DNA at 342 nm.

Table 2.4c: Influence of ionic strength on the binding of MDPSQ to calf thymus DNA

<table>
<thead>
<tr>
<th>Ionic strength of NaCl (M)</th>
<th>Adding DNA after NaCl Binding constant (M⁻¹)</th>
<th>Decrease (%)</th>
<th>Adding DNA before NaCl</th>
<th>A/A₀</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>3.28 x 10⁴</td>
<td>---</td>
<td>1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.075</td>
<td>2.84 x 10⁴</td>
<td>14.5</td>
<td>0.9421</td>
<td>8.78</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>2.59 x 10⁴</td>
<td>29.92</td>
<td>0.8925</td>
<td>13.66</td>
<td></td>
</tr>
</tbody>
</table>

A and A₀ are the absorbance of MDPSQ with and without DNA at 332 nm.
2.3.3 Fluorescence studies

Interaction of fluorescent APSQ/BAPSQ/MDPSQ with DNA induces changes in their fluorescence spectra. Fluorescence intensity of APSQ, BAPSQ and MDPSQ quenched (hypochromism) by 28, 46 and 34 % respectively upon addition of DNA, although the shape of the spectrum remained unchanged. The ratio of fluorescence intensity in the presence and in absence of DNA (I/I₀) was plotted with respect to DNA concentration to give a stern-volmer plot. Since the fluorescence intensity of DNA bound PSQ analogues is small compared to that of free PSQ analogues and the resulting Stern-volmer plot appears to be a straight line, the slope can be directly understood as the equilibrium constant for PSQ analogue-DNA complex formation. The equilibrium constant obtained were 3.81 X 10⁴ M⁻¹ (APSQ), 6.6 X 10⁴ M⁻¹ (BAPSQ) and 5.7 X 10⁴ M⁻¹ (MDPSQ) (Fig 2.3a, c, e). Benesi-Hilderbrand plot (double reciprocal plot) was constructed from changes in fluorescence intensity at 426, 432 and 465 nm respectively. Association constant for the formation of PSQ-DNA complex, was calculated from the ratio of the slope to the intercept was 1.64 X 10⁴ M⁻¹ (APSQ), 7.5 X 10⁴ M⁻¹ (BAPSQ) and 5.92 X 10⁴ M⁻¹ (MDPSQ) (Fig 2.3 b, d, f).

Since a number of drugs have been reported to exhibit sequence-specificity in binding to DNA (Waring, 1977), the interaction of APSQ, BAPSQ and MDPSQ with Clostridium perfringens DNA and Micrococcus lysodeikticus DNA were studied by Scatchard plot exhibiting a marked deviation from linearity. The intrinsic binding constant Kᵣ of 1.75 - 5.92 X 10⁴ M⁻¹ and ‘n’ number of binding sites 1.16 - 3.42 base pairs were obtained. Results are summarized in Table 2.5. Results from the stern-volmer, Benesi-Hilerbrand and Scatchard plot methods indicated that the binding of PSQ to B-form DNA is far more favourable.
Figure 2.3 A and B: (a) Stern-Volmer plot and (b) Benesi-Hilderband plot of APSQ-DNA complex.

Figure 2.3 C and D: (c) Stern-Volmer plot and (d) Benesi-Hilderband plot of BAPSQ-DNA complex.

Figure 2.3 E and F: (e) Stern-Volmer plot and (f) Benesi-Hilderband plot of MDPSQ-DNA complex.
**Table 2.5:** Summary of binding constants and exclusion parameters estimated for the interaction of APSQ, BAPSQ and MDPSQ with DNAs of varying G+C content.

<table>
<thead>
<tr>
<th>DNA</th>
<th>G+C content (%)</th>
<th>$^aK_i (10^4 M^{-1})$</th>
<th>$^b n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APSQ</td>
<td>BAPSQ</td>
<td>MDPSQ</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>28</td>
<td>1.75</td>
<td>2.29</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>42</td>
<td>3.189</td>
<td>4.16</td>
</tr>
<tr>
<td><em>Micrococcus lysodeikticus</em></td>
<td>72</td>
<td>4.517</td>
<td>5.77</td>
</tr>
</tbody>
</table>

$^aK_i$ is the apparent association constant. Binding parameters were estimated from data obtained in fluorescence titration experiments. Results were analyzed according to the neighbor exclusion model of McGhee and von-Hippel (McGhee and von-Hippel, 1974).

$^b n$ is the number of binding site size in base pairs.

**2.3.4 Viscosity Measurements**

After identification of favourable interaction that indicates probable intercalation mode of binding between PSQ analogues and DNA, viscosimetry was employed to further support this hypothesis. Molecules with intercalating ability are known to increase the length of short DNA segments on binding (Cory *et al*., 1985).

An increase in viscosity of native DNA is regarded as a diagnostic feature of an intercalation process (Muller and Crothers, 1968; Mee *et al*., 1998). A plot of relative increase in contour length $L/L_0$ versus ‘$r$’ yield slope ‘$m$’ whose values depend on the functionality of the intercalator. Monofunctional intercalators such as ethidium bromide, proflavine and aminooacridines have ‘$m$’ values between 0.8 and 1.5 while bisintercalators exhibit double this value (Le Pecq *et al*., 1975; Cohen *et al*., 1968). On plotting $L/L_0$ versus ‘$r$’ for APSQ, BAPSQ and MDPSQ (Fig 2.4a-c, Table 2.6) the resultant ‘$m$’ values were $1.02 \pm 0.04$, $1.26 \pm 0.18$ and $1.39 \pm 0.02$. 

*PSQ intercalates to DNA and induces cancer cell death*
Figure 2.4 A, B and C:
Effect of APSQ, BAPSQ and MDPSQ on the relative contour length of sonicated calf thymus DNA fragments. \( L \) represents the contour length of fragments with drug bound at the indicated binding ratio \( r \); \( L_0 \) is the contour length of control drug without DNA. The line labeled \( 1 + 2r \) represents the theoretical relation for an idealized intercalation process. The line fitted to the experimental points was computed by the method of least squares and constrained to pass through the point (0, 1). Each point represents the mean of the three experiments.
respectively (‘m’ values are compatible only with a monointercalation) indicating that these compounds provoke DNA helix extension as monofunctional intercalators. Slight higher values of BAPSQ and MDPSQ when compared to APSQ are probably the result of the additional outside binding of the dialkylamino and benzylamino side chain of MDPSQ and BAPSQ respectively to DNA helix.

**Table 2.6**: Viscosimetric measurements of APSQ, BAPSQ and MDPSQ binding to DNA.

<table>
<thead>
<tr>
<th>SI No.</th>
<th>Compound</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>APSQ</td>
<td>1.02 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>BAPSQ</td>
<td>1.26 ± 0.18</td>
</tr>
<tr>
<td>3</td>
<td>MDPSQ</td>
<td>1.39 ± 0.02</td>
</tr>
</tbody>
</table>

The slope of \( L/L_0 \) Vs \( r \) was determined by viscometry where \( L \) and \( L_0 \) are Contour length of sonicated DNA in the presence and absence of ligand and \( r \) represents the bound drug per nucleotide.

**2.3.5 Thermal Denaturation Studies**

Thermal denaturation study of DNA-PSQ analogue complexes provided the additional strong evidence for the intercalative ability of PSQ analogues (APSQ, BAPSQ and MDPSQ) into double helix DNA. Intercalation of small molecules into the helix is known to increase DNA melting temperature (\( T_m \)) at which double stranded DNA denatures into single stranded DNA (helix to coil transition), due to the increased stability of the helix (Cao and He, 1998). Thermal denaturation of DNA show an increase in the absorbance at 260 nm indicating that the extinction coefficient of dsDNA is less than ssDNA which is in turn lesser than free nucleotides (Zuby, 1988). Thus the \( T_m \) can be determined by a plot of DNA absorbance at 260 nm as a function of temperature. \( T_m \) of different DNA with increasing G+C content was 62°, 65° and 71° respectively. On complexing with PSQ analogues the \( T_m \) increased in the range of 2.9° to 6.8°, with single transition for all three molecules as shown in
Table 2.7: DNA with higher G+C content did show higher \( T_m \) value. These results strongly supported the intercalation of PSQ analogues into double helix DNA.

**Table 2.7:** \( \Delta T_m \) of the pyrimidoselenoloquinolines with different G+C containing DNAs

<table>
<thead>
<tr>
<th>DNA</th>
<th>G+C Content (%)</th>
<th>( \Delta T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Clostridium perfringens )</td>
<td>28</td>
<td>APSQ 2.9, BAPSQ 3.4, MDPSQ 3.8</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>42</td>
<td>APSQ 4.1, BAPSQ 6.0, MDPSQ 5.0</td>
</tr>
<tr>
<td>( Micrococcus lysodeikticus )</td>
<td>72</td>
<td>APSQ 4.6, BAPSQ 6.8, MDPSQ 6.0</td>
</tr>
</tbody>
</table>

\( \Delta T_m \) (Melting temperature) = \( (T_0 - T) \); where \( T_0 = T_m^{\text{drug-DNA complex}} \), and \( T = T_m^{\text{DNA alone}} \)

**2.3.6 Circular Dichroism Studies**

Intercalation of a planar aromatic molecule into B-DNA causes extension and local unwinding of the polynucleotide double helix (Lerman, 1961). Thus, in the complex region, the relative positions of the bases are substantially changed. The modified structure containing the planar ligand, besides affecting the hydrodynamic properties of DNA, dramatically alters the chiroptical response of the polynucleotide, leading to significant effects in their CD spectra (Kapuscinski and Darzynkiewicz, 1987; Dourlent and Helene, 1971; Fasmann 1996; Palumbo et al., 1985).

In presence of PSQ analogues the CD spectra of DNA double helix was perturbed. Figure 5a-c presents the CD changes on the interaction of PSQ analogues to DNA. The uncomplexed calf thymus DNA displayed a positive signal at 250 nm and a negative signal at 280 nm, which are typical features of B-DNA (Mauffret et al., 1989). On addition of PSQ analogues to a solution of DNA the negative band decreased gradually, whereas the positive signal showed hypochromism (37-58%) with a red shift (\( \Delta \lambda \approx 3 \text{nm for negative peak and } \Delta \lambda \approx 11 \text{ nm for positive peak} \)). Such changes were likely to result from structural alterations induced by PSQ analogues into the double-helical structure. It has been assumed that a CD shift with magnitude
Figure 2.5 A and B:
CD spectra of calf thymus DNA titrated with (a): APSQ and (b): BAPSQ at (1) $r' = 0$; (2) 0.06; (3) 0.15; (4) 0.20; (5) 0.27; (6) 0.33.

Figure 2.5 C:
CD spectra of calf thymus DNA titrated with MDPSQ at (1) $r' = 0$; (2) 0.06; (3) 0.15; (4) 0.20; (5) 0.27; (6) 0.33.
equal to $\Delta \lambda \geq 15$ nm is observed consecutively to a chromophore intercalation (Dougherty and Pigram, 1982). Invoked reasons are that the compound-base stackings and the dipole-dipole interactions are stabilizing the intercalated compound in the helix, results in a shift to a larger wavelength and hypochromism (Dougherty and Pigram, 1982; Berman and Young, 1981). These effects are, thus similar to those displayed by our complexes.

Further the complexation of BAPSQ / MDPSQ with DNA leads to an extrinsic positive CD band at 325 and 350 nm respectively, i.e., the main UV absorption signal of PSQ but alongside the DNA spectrum. Compared to the free drug, these demonstrate both hyperchromism and red shift ($\Delta \lambda \approx 4$ nm) compatible with intercalation. Although varied intermediates may concern the intercalation of PSQ analogues into base pairs, the present findings permit one to distinguish between two extreme arrangements; the drug lies within the intercalation site with the long direction of its chromophore oriented either parallel (classical model denoted 'A' and characterized by a negatively induced CD) or perpendicular (threading model denoted 'B' and characterized by a positively induce CD) to the base pair length axis (Fig 2.6 A-B). Accordingly, the positive sign of the induced CD band at 325 and 350 nm for BAPSQ and MDPSQ may implicate intercalation, as shown in model 'B'. The alternative model 'A' would result in a negatively induced CD band. The present results match the structural model 'B'.

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Figure 2.6 A: Model of PSQ intercalation into base pairs. In this case the chromophore is perpendicular to the DNA axis with its long direction, parallel in ‘A’ to the base-pair long axis \( (r = 90^\circ) \).

Figure 2.6 B: Model of PSQ intercalation into base pairs. In this case the chromophore is perpendicular to the DNA axis with its long direction, perpendicular in ‘B’ to the base-pair long axis.
2.4. Discussion

Based on our previous studies and various other reports, observing the chemical structure an intercalative binding mechanism might be reasonably expected for all three PSQ analogue molecules considered in this work.

Qualitative and quantitative aspects of the interaction between PSQ analogues and DNA have been studied spectrophotometrically by utilizing alterations in the PSQ analogues spectrum that result from complex formation. The PSQ analogues used in the present study exhibits different levels of DNA binding. Intercalation results in significant changes in its UV-visible spectra, typically exhibiting a red shift and substantial hypochromicity.

Sodium chloride affected the binding of PSQ analogues to native DNA. The influence of ionic environment on the ability of PSQ analogues to bind to DNA indicated that electrostatic factors are important in complex formation. Presumably ionic attraction between the positively charged PSQ molecules and anionic phosphate groups of DNA supplements the more specific forces of attraction which bind the ligand to either purine or pyrimidine moieties. Results suggested that binding involves both electrostatic attraction between the protonated ring system of PSQ analogues and the anionic phosphate groups of DNA and a more specific interaction apparently involving the aromatic ring portions of PSQ analogues and nucleotide bases.

Complexing of PSQ analogues with DNA varying in GC content resulted in quenched fluorescence and formation of curved plots of r/C versus r that yielded slightly higher values for ‘n’ and ‘K_i’ with increasing GC composition. Since the amount of spectral shift increased with the GC content of the polymer and the hypochromicity associated with the ligand binding to pyrimidine residues was
relatively small, it appears that the differences observed with DNA preparations from
different sources represent primarily a greater perturbation effect of guanine on the
PSQ spectrum as compared with adenine. Such an effect may be related to the
demonstrated relative superiority of guanine as an electron donor (Pullman and
Pullman, 1958). It should also be noted that a G:C preference has already been shown
for a number of antitumor intercalating drugs of clinical importance, including
Actinomycin D (Muller and Crothers, 1968), mitoxantrone (Lown, 1983),
Daunomycin (Chaires et al., 1982), 2-N-methyl-9-hydroxellipticinium (Schwaller et
al., 1990).

Increase in viscosity of the short DNA segments (increased contour length
L/L₀) observed for PSQ analogues (as a result of the reduced flexibility of the helix
when drug is intercalated) follows the expected pattern for intercalation. Its value is
compatible with monointercalation. Little higher values of BAPSQ and MDPSQ are
probably the result of rigidification of the DNA helix caused by the binding of the
side chain on the outside. Intercalators increase the stability of the helix (Cao and He,
1998) thus increasing the Tm, temperature required to denature double stranded DNA
to single stranded DNA. The results obtained with our intercalating compounds are
totally consistent with the above findings.

Although the CD spectrum of drugs that bound to DNA is not fully
understood, it is known to be very sensitive to the environment of the drugs. The
appearance of the CD spectra reflects the binding geometry and binding mode of the
drug as well as the arrangement of neighboring bases (Lee et al., 2001). Decrease in
molar absorptivity at 280 nm and emergence of induced CD within the red shifted
absorption band (Δλ ≥ 11, from the free drug band) during an achiral chromophore-
DNA interaction reflects undoubtedly intercalation. However several geometries are possible, according to the orientation of chromophores about the base pairs. Discrimination between intercalative geometries is needed for a better understanding of the biological properties of intercalators especially those bearing functional groups on the chromophore which because of their orientation, way determine their interactions with the proteins. In model ‘A’ functional group of intercalators appear buried in the DNA double helix and thus is less accessible to the enzyme. In model ‘B’ the group protrudes into one of the DNA grooves where it is able to interact freely with the enzyme. Intercalative model ‘B’ is therefore more apt than model ‘A’ to explain the cytotoxicity of PSQ.

The success of this study relative to the significance of CD data for the mode and the conformational effects of PSQ –DNA interactions is based on the information obtained from three sources; the occurrence of an induced CD band alongside the DNA spectrum, the sign of this induced CD band and the specific variations in the CD spectrum of DNA.
2.5 Conclusion

PSQ-DNA complex formation resulted in induction of bathochromicity and hypochromicity in the ligand absorption bands and dramatic changes in drug fluorescence quantum yields, enhancement in specific viscosity of sonicated rodlike nucleic acid, increase in melting temperature of the macromolecule and large CD perturbations along with emergence of induced CD with red shift should be considered as good evidence for intercalation of the molecule into a particular DNA site. These findings were used altogether as reasonably safe probes for an intercalative binding mechanism even though none of these lines of evidence constitutes by itself a firm diagnostic tool. All three PSQ analogues satisfy the above requirements. Based on these results we conclude that APSQ, BAPSQ and MDPSQ molecules intercalate into DNA.