CHAPTER II

Experimental
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EXPERIMENTAL METHODS

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1. Materials
CuCl₂·2H₂O, Cu(NO₃)₂·3H₂O, ZnCl₂, Zn(NO₃)₂·6H₂O (Merck), SnCl₄·5H₂O, ZrCl₄ (Lancaster), (CH₃)₂SnCl₂ (Sigma), 2-amino-3-formylchromone (Aldrich), L-Valine (Lobachem), L-Alanine, L-Proline, N-Hydroxybenzotriazole (HOBT) (SRL), Boc anhydride, SOCl₂, Trifluoroacetic acid (TFA), (R)/(S)-2-amino-2-phenylethanol, (R)/(S)-2-amino-1-propanol, Tris(hydroxymethyl) aminomethane (Tris-buffer), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluraniumpentafluoro phosphate (HBTU) (Sigma), Boc-L-Valine, Dicyclohexylcarbodiimide (DCC) (Fluka) were used as received. Disodium salt of calf thymus DNA (CT-DNA), guanosine 5′-monophosphate disodium salt (5′-GMP) and thymine 5′-monophosphate disodium salt (5′-TMP) were purchased from Sigma and stored at 4 °C. 6X loading dye (Fermental Life Science), agarose gel, ascorbic acid, sodium azide (NaN₃), DMSO, superoxide dismutase (SOD), methyl green, DAPI, mercaptopropionic acid (MPA) (Sigma-Aldrich) and supercoiled pBR322 DNA (Genei) were utilized as received. All reagent grade compounds were used without further purification.

2. Characterization techniques
Carbon, hydrogen and nitrogen contents were determined using Carlo Erba analyzer model 1108. Melting points were determined by electrothermal apparatus. TLC was performed on commercial aluminium sheets precoated with 0.20 mm layer of silica gel. The visualization of spots on TLC plates was effected by exposure to iodine and ninhydrin.

2.1 Infrared spectroscopy
Fourier transform infrared spectra were recorded on Interspec 2020 FTIR spectrometer. The infrared spectroscopy is a useful technique to characterize a compound. It results
from transition between vibrational and rotational energy levels. IR region of the electromagnetic spectrum covers a wide range of wavelength from 200-4000 cm\(^{-1}\). It has been found that in IR absorption, some of the vibrational frequencies are associated with specific groups of atoms and remain same irrespective of the molecules in which the group is present. These are called characteristic frequencies and their constancy results from the constancy of bond force constants from molecule to molecule. The important observations that the IR spectrum of a complex molecule consists of characteristic group frequencies makes IR spectroscopy, unique and powerful tool in structural analysis.

2.2 Ultra-violet and visible spectroscopy

The electronic spectral studies of metal complexes provide useful information about the stereochemistry, oxidation state of the metal ion and in suitable circumstances, the nature of metal-ligand bond. Electronic spectra were recorded on a UV-1700 PharmaSpec UV–visible spectrophotometer in methanol and DMSO as solvent.

2.3 Nuclear magnetic resonance spectroscopy

The nuclei of certain isotopes possess a mechanical spin or angular momentum. The NMR spectroscopy is concerned with nuclei having nuclear spin quantum number \( I = 1/2 \), example of which include \(^1\)H, \(^{13}\)C, \(^{31}\)P, \(^{119}\)Sn and \(^{19}\)F.

For a nucleus with \( I = 1/2 \), there are two values for the nuclear spin angular momentum quantum number \( m_I = \pm 1/2 \) which are degenerate in the absence of a magnetic field. However, in presence of the magnetic field, this degeneracy is destroyed such that the positive value of \( m_I \) corresponds to the lower energy state and negative value to higher energy state separated by \( \Delta E \).
In an NMR experiment, one applies strong homogeneous magnetic field causing the nuclei to precess. Radiation of energy comparable to $\Delta E$ is then imposed with radio frequency transmitter equal to precision or Larmor frequency and the two are said to be in resonance. The energy can be transferred from the source to the sample. The NMR signal is obtained when a nucleus is excited from low energy to high energy state. $^1$H, $^{13}$C and $^{119}$Sn NMR spectra were obtained on a Bruker Avance DRX-400 spectrometer at 400 and 500 MHz, respectively operating at room temperature.

2.4 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) spectra of the Cu(II) complexes were obtained on a Varian E 112 EPR spectrometer using tetracyanoethylene (TCNE) as field marker. The spectra were recorded for solutions of the complexes in appropriate solvents at liquid nitrogen temperature (LNT).

2.5 Mass spectrometry

Mass spectrometry is one of the most accurate micro analytical techniques which require only a few nano moles of the sample to obtain characteristic information regarding the molecular mass and to detect within a molecule the places at which it prefers to fragment from which the presence of recognizable group within the molecule can be deduced. Mass spectrometry is complementary to FTIR, NMR, UV–vis and EPR spectral techniques for structural identification of compounds. Electrospray mass spectra were recorded on Micromass Quattro II triple quadrupol mass spectrometer.
2.6 Molar conductance measurements

The conductivity measurement is one of the simplest and easily available techniques used to study the nature of the complexes. It gives direct information regarding whether a given compound is ionic or covalent.

For this purpose, the measurement of molar conductance (\( \Lambda_m \)), is calculated using the equation;

\[
\Lambda_m = \frac{\text{cell constant x conductance}}{\text{concentration of solute expressed in mol cm}^{-3}}
\]

Conventionally, solutions of 1.0x10^{-3} M strength are used for the conductance measurements. Molar conductance was measured at room temperature on a Digisun electronic conductivity bridge.

2.7 Polarimetry

Optical isomerism manifests itself by the rotation that certain molecules impart to the plane of polarized light when in gaseous, liquid or molten state or in solution (Figure 20). This rotation is observed and measured by a rather simple instrument, known as polarimeter. The specific rotation \([\alpha]\) of a dissolved substance is given by the expression;

\[
[\alpha] = \frac{\alpha}{l \times c}
\]

where

- \( \alpha \) is the observed rotation in degrees
- \( l \) is the path length of the sample in decimeters
- \( c \) is the concentration in grams per milliliter
The dependence on wavelength and temperature is indicated by subscripts and superscripts respectively. Thus \([\alpha]_D^{25}\) means the specific rotation at 25 °C measured at the wavelength of the sodium D line.

Optical rotation is generally measured using light from a sodium-vapour lamp, which gives essentially monochromatic radiation (the yellow sodium D line is a doublet at 5890 and 5896 Å).

**Figure 20. Rotation of plane of polarized light by optically active compounds.**

A beam of light is polarized by the passage through nicol prism (the polarizer), which consists of two calcite prisms cemented together so that only one of the two rays formed by double refraction is transmitted. The beam of polarized light passes through the solution and then through a second nicol prism. When no optically active material is placed between the prisms (0° rotation), the prisms are positioned at right angles so that no light is transmitted. When an optically active material is placed between the prisms, the analyzer must be turned in order to maintain the darkness in the field of view. The optical rotation is the angle by which the analyzer is turned in order to reach darkness. It is very difficult to determine by eye the setting for complete darkness, because positions near the completely dark position are very dark. Therefore, many instruments are
constructed such that the field of view is divided into two equal parts, and the analyzer is adjusted so as to equalize the light intensity in each half of the field.

Optical rotations of chiral ligands and complexes were determined on a Polarimeter Rudolf Autopol III.

3. DNA binding studies

All the experiments involving interaction of the complexes with CT-DNA were conducted in Tris buffer containing HCl (0.01 M) adjusted to pH 7.2 with hydrochloric acid. The CT-DNA was dissolved in Tris-HCl buffer and was dialyzed against the same buffer overnight. Solutions of CT-DNA gave ratios of UV absorbance at 260 and 280 nm above 1.8, indicating that the DNA was sufficiently free of protein [170]. DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient 6600 dm$^3$ mol$^{-1}$ cm$^{-1}$ at 260 nm [171]. The stock solution was stored at 4 °C.

3.1 Absorption spectral studies

The intrinsic binding constant $K_b$ of the complex to CT-DNA was determined by equation (1), from the plot of [DNA]/|$\varepsilon_a$-$\varepsilon_f$| vs. [DNA], where [DNA] represents the concentration of DNA, and $\varepsilon_a$, $\varepsilon_f$, and $\varepsilon_b$ the apparent extinction coefficient ($A_{obs}$/[M]), the extinction coefficient for free metal complex (M), and the extinction coefficient for the complex (M) in the fully bound form, respectively. In plots of [DNA]/|$\varepsilon_a$-$\varepsilon_f$| vs. [DNA], $K_b$ is given by the ratio of slope to intercept [172].

$$[\text{DNA}]/|\varepsilon_a$-$\varepsilon_f| = [\text{DNA}]/|\varepsilon_b$-$\varepsilon_f| + 1/K_b|\varepsilon_b$-$\varepsilon_f|$$  \hspace{1cm} (1)

Absorption spectral titration experiments were performed by maintaining a constant concentration of the complex and varying the nucleic acid/nucleotide concentration.
This was achieved by diluting an appropriate amount of the metal complex solutions and DNA/5′-GMP and 5′-TMP stock solutions while maintaining the total volume constant. This results in a series of solutions with varying concentrations of DNA/5′-GMP and 5′-TMP but a constant concentration of the complex. The absorbance (A) was recorded after successive additions of DNA or 5′-GMP or 5′-TMP. While measuring the absorption spectra an equal amount of either CT-DNA or 5′-GMP or 5′-TMP was added to both the compound solution and reference solution to eliminate the absorbance of either CT-DNA or 5′-GMP or 5′-TMP itself.

3.2 Fluorescence spectral studies

The emission spectrum is obtained by setting the excitation monochromator at the maximum excitation wavelength and scanning with emission monochromator. Often an excitation spectrum is first made in order to confirm the identity of the substance and to select the optimum excitation wavelength. Further experiments were carried out to gain support for the mode of binding of complexes with CT-DNA. Non-fluorescent or weakly fluorescent compounds can often be reacted with strong fluorophores enabling them to be determined quantitatively. On this basis molecular fluorophore EB was used which emits fluorescence in presence of CT-DNA due to its strong intercalation. Quenching of the fluorescence of EB bound to DNA were measured with increasing amounts of metal complexes as a second molecule and Stern-Volmer quenching constant K was obtained from the following equation (2) [173].

\[
\frac{I_o}{I} = 1 + Kr
\]  

(2)

Where, r is the ratio of total concentration of complex to that of DNA and \(I_o\) and I are the fluorescence intensities of EB in the absence and presence of complex. Binding
constant K of the metal complexes was also determined from equations (3) and (4) (Scatchard equation) by emission titration [174,175].

\[ C_F = C_T (I/I_0-P) (1-P) \]  \hspace{1cm} (3)
\[ r/C_F = K (n-r) \]  \hspace{1cm} (4)

Where \( C_F \) is the free probe concentration, \( C_T \) is the total concentration of the probe added, \( I \) and \( I_0 \) are fluorescence intensities in presence and absence of CT-DNA, respectively and \( P \) is the ratio of the observed fluorescence quantum yield of the bound probe to that of the free probe. The value \( P \) was obtained as the intercept by extrapolating from a plot of \( I/I_0 \) vs \( 1/[DNA] \), \( r \) denotes ratio of \( C_B \) (\( C_B=C_T-C_F \)) to the DNA concentration i.e., the bound probe concentration to the DNA concentration, \( K \) is the binding constant and \( C_F \), is the free metal complex concentration and “n” is the binding site number. Emission intensity measurements were carried out using Hitachi F-2500 spectrofluorometer at room temperature.

### 3.3 Circular dichroic spectral studies

Circularly polarized light represents a wave in which the electrical component spirals around the direction of propagation of the ray, either clockwise or counterclockwise. Within the absorption band, the molar absorptivity for right and left handed circularly polarized light is different, that is \( (\varepsilon_d-\varepsilon_l) \neq 0 \). This effect changes linearly polarized light into elliptically polarized light and is known as circular dichroism. The amplitude of d component will be greater than the l component when l component of the substance absorbs left circularly polarized light, more strongly than d component which absorb the right circularly polarized light i.e. \( \varepsilon_l > \varepsilon_d \). Furthermore, if \( \varepsilon_d > \varepsilon_l \) then the d component will be retarded more than the l component (Figure 21).
The ellipticity, that is the angle whose tangent is ratio of minor axis of the ellipse OB to the major axis OA, is denoted by \( \theta \). The molecular ellipticity \([\theta]\) can be shown by the relationship [176].

\[
[\theta] = 3305 (\epsilon_l - \epsilon_d)
\]

Circular dichroism graphs are plots of \([\theta]\) against wavelength. Circular dichroic spectra were obtained on Applied Photophysics Chirascan Circular Dichroism Spectrometer with stop flow.

![Diagram of elliptically polarized light](image)

**Figure 21.** Elliptically polarized light produced when \( \epsilon_d > \epsilon_l \) and \( \epsilon_l > \epsilon_d \).

### 3.4 Viscometric studies

The hydrodynamic changes are the consequence of the change in length of the molecule, the diminished bending between layers and the diminished length-specific mass. Flow time was measured with a digital stopwatch. Each sample was measured three times and an average flow time was calculated. Data were presented as \( (\eta/\eta_0) \) versus binding ratio ([M]/[DNA]), [177,178] where \( \eta \) is viscosity of DNA in the presence of complex and \( \eta_0 \) is the viscosity of DNA alone. Viscosity values were calculated from the observed flow
time of DNA containing solution \((t>100\text{s})\) corrected for the flow time of buffer alone \((t_0)\), \(\eta = t-t_0\).

Viscosity measurements were carried out using Ostwald capillary viscometer maintained at \(25\pm0.01 \, ^\circ\text{C}\). Several time readings were obtained at each titration point.

### 3.5 Gel electrophoresis

Gel electrophoresis is a technique widely used for separation and analysis of charged biomolecules like nucleic acids [179]. Any charged biomolecule migrates when placed in an electric field. The ratio of migration of a molecule depends on its net charge, size, shape and the applied current. This can be represented as follows:

\[
V = E \cdot \frac{q}{f}
\]

Where \(V\) = velocity of migration of the molecule, \(E\) = electric field in volts/cm, \(q\) = net charge on the molecule, \(f\) = frictional coefficient which is function of mass and shape of molecule. The movement of a charged molecule in an electric field is often expressed in terms of electrophoretic mobility \((\mu)\), which is defined as the velocity per unit of electric field.

\[
\mu = \frac{V}{E} = E \cdot \frac{q}{f}
\]

\[
\mu = \frac{q}{f}
\]

For molecules with similar conformation, \(f\) varies with size but not with shape. Thus electrophoretic mobility \((\mu)\) of a molecule is directly proportional to the charge density (charge/mass ratio). Molecules with different charge/mass ratio migrate under the electric field at different rates and hence get separated. This is the basic principle for all the electrophoretic techniques. Depending upon the nature of support medium, electrophoresis is of different types such as paper, starch, polyacrylamide, and agarose.
gel electrophoresis. We have opted agarose gel electrophoresis, because agarose gels are more porous as compared to polyacrylamide gels and are, therefore, used to fractionate large macromolecules such as DNA that cannot be readily penetrate into and move through other types of supporting materials. Agarose is a linear polymer of D-galactose and 3,6-anhydro-L-galactose. When an electric field is applied across agarose gel, DNA molecules that are negatively charged at neutral pH, migrate towards oppositely charged electrode at rates determined by their molecular size and conformation. DNA molecules of the same size but with different conformation travel at different rates.

The order of migration velocity in the increasing order of various forms of DNA is: supercoiled DNA>linearized DNA>open circular DNA.

4. Computational studies

Molecular docking is carried out using a computer program in order to dock computer-generated representations of small molecules to a receptor (or to a user-defined part thereof, e.g. the active site of an enzyme), followed by evaluation of the molecules with respect to complementarity in terms of shape and properties, such as electrostatics. Good complementarity of a molecule indicates that the molecule is potentially a good binder. The outcome of a docking exercise normally includes some sort of affinity prediction for the molecules investigated, yielding a relative rank-ordering of the docked compounds with respect to affinity.

The rigid molecular docking study performed by using HEX 6.1 software [180], is an interactive molecular graphics program for calculating and displaying feasible docking modes of a pair of protein and DNA molecule. Structure of the complex was sketched by CHEMSKETCH and converted into ‘pdb’ format from ‘mol’ format by molecular
format converter by online OPENBABEL. The crystal structure of the B-DNA
dodecamer d(CGCGAATTCGCG)$_2$ (PDB ID: 1BNA) was downloaded from the protein
data bank. Visualization of the docked pose has been done by using CHIMERA
molecular graphics program.

5. In vitro antitumor studies

The cell lines used for in vitro antitumor screening activity were A498 (Renal Cell),
A549 (Lung), Zr-75-1 (Breast), HT29 (Colon adenocarcinoma grade II cell line), A2780
(Ovary), SiHa (Uterine Cervix) and MCF7 (Human breast). These human malignant cell
lines were procured and grown in RPMI-1640 medium supplemented with 10% Fetal
Bovine Serum (FBS) and antibiotics to study growth pattern of these cells. The
proliferation of the cells upon treatment with chemotherapy was determined using the
Sulforhodamine B (SRB) semi automated assay [181]. The dose response parameters
such as growth inhibition 50% (GI$_{50}$), total growth inhibition (TGI) and lethal
concentration 50% (LC$_{50}$) were calculated. GI$_{50}$ is the concentration of drug required to
decrease the cell growth to 50%, compared with that of the untreated cell number. TGI
is the concentration of drug required to decrease the cell growth to 100%, compared
with that of the untreated cell number, during drug incubation. LC$_{50}$ is the concentration
of drug required to decrease the cell growth by 50% of the initial cell number prior to
the drug incubation. Cells were seeded in 96 well plates at an appropriate cell density to
give optical density in the linear range (from 0.5 to 1.8) and were incubated at 37 °C in
CO$_2$ incubator for 24 h. Stock solutions of the complexes were prepared as 100 mg/ml
in DMSO and four dilutions i.e. 10 µL, 20 µL, 40 µL, 80 µL, in triplicates were tested,
each well receiving 90 µL of cell suspension and 10 µL of the drug solution.
Appropriate positive control (Adriamycin) and vehicle controls were also run. The plates with cells were incubated in CO\(_2\) incubator with 5% CO\(_2\) for 24 h followed by drug addition. The plates were incubated further for 48 h. Termination of experiment was done by gently layering the cells with 50 µL of chilled 30% TCA (in case of adherent cells) and 50% TCA (in case of suspension cell lines) for cell fixation and kept at 4 °C for 1 h. Plates were stained with 50 µL of 0.4% SRB for 20 min. The bound SRB was eluted by adding 100 µL 10 mM Tris (pH 10.5) to each of the wells. The absorbance was read at 540 nm with 690 nm as reference wave length. All the experiments were repeated 3 times.