Experimental
EXPERIMENTAL METHODS

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1. Materials
CuCl₂.2H₂O, ZnCl₂, 2-amino-benzothiazole and dibromoethane (Merck), oxalyl chloride (Fluka), SnCl₄.5H₂O (Lancaster), salicylaldehyde (Alfa Aesar), 2-amino-6-fluorobenzothiazole, Dimethyltin(IV) dichloride, Diphenyltin(IV) dichloride, L/D-valine, tris(hydroxymethyl)aminomethane or Tris Buffer, agarose, ascorbic acid, sodium azide (NaN₃), DMSO, superoxide dismutase (SOD), methyl green, DAPI, mercaptopropionic acid (MPA) (Sigma Aldrich). Disodium salt of calf thymus DNA (CT DNA) was purchased from Sigma & Co and was stored at −20 °C. 6X loading dye (Fermental Life Science) and supercoiled plasmid DNA pBR322 (Genei) were utilized as received. All reagents were of the best commercial grade and were used without further purification.

2. Characterization techniques
Carbon, hydrogen and nitrogen contents were determined using Carlo Erba Analyzer Model 1106. The visualization of spots on TLC plates was effected by exposure to iodine or spraying with 10% H₂SO₄ and charring and single spots of the products were observed which were different from those of the starting materials.

2.1 Infrared spectroscopy
Infra-red spectra of the ligands and complexes were recorded as KBr pellets on Interspec 2020 FTIR spectrometer. The formation of the ligands and complexes behavior has been ascertained by scanning their infrared spectra in 4000–400 cm⁻¹.

2.2 Ultraviolet 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 UV–1700 PharmaSpec UV–vis spectrophotometer (Shimadzu). Data were reported in λmax/nm.

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 ¹H, ¹³C and ¹¹⁹Sn.
For a nucleus with I = 1/2, there are two values for the nuclear spin angular momentum quantum number m_I = ±1/2 which are degenerate in the absence of a magnetic field. However, in the presence of the magnetic field, this degeneracy is destroyed such that the
positive value of \( m_1 \) 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 press. 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.

The \(^1\)H, \(^{13}\)C and \(^{119}\)Sn NMR spectra were obtained on a Bruker DRX-400 spectrometer at 400, 100 and 146 MHz, respectively operating at room temperature. Chemical shifts were reported on the \( \delta \) scale in parts per million (ppm).

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 solid and 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 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.

ESI mass spectra is relatively routine technique for mass spectrometric analysis of compounds whether charged or neutral but involves complication by redox, fragmentation and clustering processes in the study of metal complexes. ESI mass spectra were recorded on a Micromass Quattro II triple quadrupol mass spectrometer.

2.6. Thermal gravimetric analysis (TGA)

The TGA analysis of complexes was performed on Shimadzu DTG–60H analyzer under nitrogen atmosphere from room temperature to 1000 °C at a heating rate of 20 °C/min. The final thermolysis product of metal complex as metal oxides was studied.
2.7. 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 (1);

$$\Lambda_m = \frac{\text{Cell constant} \times \text{conductance}}{\text{Concentration of solute expressed in mol cm}^{-3}}$$

(1)

Conventionally, solutions of $10^{-3}$ M strength are used for the conductance measurements. Molar conductance was measured at room temperature on a Digisun electronic conductivity bridge.

2.8. Magnetic measurements
Magnetic susceptibility measurements were determined at 295 K with Sherwood scientific magnetic susceptibility balance as depicted in Figure 23.

![Sherwood scientific magnetic susceptibility balance](image)

**Figure 23. Sherwood scientific magnetic susceptibility balance.**

The effective magnetic moment was calculated by using following equations (2-4):

$$\chi_g = \frac{C L (R - R_o)}{10^9 M}$$

(2)

Where, $C$ is constant equal to 1, $R$ is the reading of the sample, $L$ is the length of sample taken (measured manually by scale), $R_o$ is the volume susceptibility, $R_o$ is the reading of blank tube and $M$ is the mass of the sample.
The effective magnetic moment was calculated by the equation:

\[ \mu_{\text{eff}} = 2.828[\chi_m T]^{1/2} \tag{4} \]

3. DNA binding studies

All the experiments involving interaction of the complexes with CT DNA were conducted in buffer containing Tris(hydroxymethyl)aminomethane or Tris Buffer (0.01 M) adjusted to pH 7.2 with 4M 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 nm in 1.9:1 ratio, 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 dm³ mol⁻¹ cm⁻¹ at 260 nm [171]. The stock solution was stored at 4 °C.

3.1 Absorption spectral studies

Absorption spectral studies were performed on Shimadzu UV–1700 PharmaSpec UV–vis spectrophotometers. The intrinsic binding constant \( K_b \) of the complex to CT DNA was determined from equation (5), through a plot of \([\text{DNA}]c_{\alpha-e_f} vs \text{[DNA]}\), where \([\text{DNA}]\) represents the concentration of DNA, and \( c_{\alpha}, e_f \) and \( e_b \) the apparent extinction coefficient \( (A_{obs}/[M]) \), the extinction coefficient for free metal complex \( (M) \), and the extinction coefficient for the free metal complex \( (M) \) in the fully bound form, respectively. In plots of \([\text{DNA}]e_{\alpha-e_f} vs \text{[DNA]}\), \( K_b \) is given by the ratio of slope to intercept [172].

\[
\frac{[\text{DNA}]}{e_{\alpha-e_f}} = \frac{[\text{DNA}]}{e_b-e_f} + \frac{1}{K_b(e_{\alpha-e_f})} \tag{5}
\]

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

3.2 Fluorescence spectral studies

Emission intensity measurements were carried out using RF-5301 PC spectrofluorophotometer (Schimadzu) in a 1 cm path—length quartz cell. The emission spectrum is obtained by setting the excitation monochromatic at the maximum excitation wavelength and scanning with emission monochromatic. 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 EthBr was used which emits fluorescence in presence of CT DNA due to its strong intercalation. Quenching of the fluorescence of EthBr bound to DNA were measured with increasing amount of metal complexes as a second molecule and Stern—Volmer quenching constant K was obtained from the following equation (6) [173]:

\[
\frac{I_0}{I} = 1 + Kr
\]

(6)

Where \( r \) is the ratio of total concentration of complex to that of DNA and \( I_0 \) and \( I \) are the fluorescence intensities of EthBr in the absence and presence of complex.

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 in the 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 \( I/1000 \) vs \( I/[DNA] \), \( r \) denotes ratio of \( C_B (=C_T-C_P) \) 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.

Binding constant K of the metal complexes was also determined from Scatchard equations (7) and (8) by employing emission titration [174,175].

\[
C_F = C_T(I/I_0-P)(1-P)
\]

(7)

\[
r/C_F = K(n-r)
\]

(8)
3.3. Circular dichoric 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, i.e. $(\varepsilon_d - \varepsilon_l) \neq 0$. 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 24).

![Diagram](attachment:ellipse.png)

**Figure 24. Elliptically polarized light produced when $\varepsilon_d > \varepsilon_l$ and $\varepsilon_l > \varepsilon_d$**

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].

$$[\Theta] = 3305 (\varepsilon_l - \varepsilon_d) \quad (9)$$

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

3.4. Agarose-gel electrophoresis

Gel electrophoresis is a technique widely used for separation and analysis of charged biomolecules like nucleic acids [177,178]. 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:

\[ V = \frac{E \cdot q}{f} \quad (10) \]

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.

\[ \mu = \frac{E \cdot q}{f \cdot E} = \frac{q}{f} \quad (11) \]

\[ \mu = \frac{q}{f} \quad (12) \]

For molecules with similar conformation, \( f \) varies with size but not with shape.

**Figure 25.** Agarose gel electrophoresis migration showing different forms of pBR322 plasmid DNA in presence of drug.

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 readily penetrate into and move through
other types of supporting materials (Figure 25). 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.

The cleavage experiments of supercoiled pBR322 DNA (300ng) in (5mM Tris–HCl, 50mM NaCl), buffer at pH 7.4 was carried out using agarose gel electrophoresis. The samples were incubated for 1 hour at 37 °C. A loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol was added and electrophoresis was carried out at 60 V for 1 hour in Tris–HCl buffer using 1% agarose gel containing 1.0 μg/mL ethidium bromide. The DNA cleavage with added reductant was monitored as in case of cleavage experiment without added reductant using agarose gel electrophoresis. The reaction were also monitored upon addition of various radical inhibitors such as sodium azide (NaN₃), DMSO, superoxide dismutase (SOD), distamycin, methyl green and mercaptopropionic acid (MPA). The samples were incubated for 45 minutes at 37 °C. The gel was visualized by photographing the fluorescence of intercalated ethidium bromide under a UV illuminator. The cleavage efficiency was measured by determining the ability of the complex to convert the supercoiled DNA (SC) to nicked circular form (NC) and linear form (LC).

4. Antimicrobial assay

Antimicrobial screening of the complexes was performed by the Agar well diffusion method of Perez et al. [179], also described earlier by Ahmad et al. [180]. 0.1 ml of diluted inoculums (10⁵c.f.u./ml) test organism was spread on nutrient agar (NA)/SD plates. Wells of 8 mm diameter were punched into the agar medium and were filled with test solutions of different concentrations (100 mg/ml, 200 mg/ml and 400 mg/ml), solvent blank (DMSO) and antibacterial drug (nitrofurantoin, 100 mg/ml). The plates were incubated for 18 h at 37 °C for test bacteria viz., B. subtilis, S. aureus, E. coli, P. aeruginosa and pathogenic fungus C. albicans. The antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism.
5. MTT Cytotoxicity assay

Human breast adenocarcinoma cell line (MDA-MB 231) and human malignant melanoma cell line (A375) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and supplemented with 10% heat inactivated foetal bovine serum. Human colon carcinoma cell line (HCT116) was cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37 °C in presence of 5% CO₂.

The MTT assay was used as a relative measure of cell viability. Briefly, cells were seeded at the density of 2 x 10⁴ cells/ml. Quadruple cell samples were grown in 96 well micro-titer plates (Iwaki, Tokyo, Japan). After 24 h, samples were exposed to different concentrations of complexes (1.56–100 μM) in a final volume of 100 μL of culture medium. Cells were incubated for 72 h in a humidified atmosphere of 5% CO₂ at 37 °C. At the end of incubation, each well received 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in phosphate-buffered saline, PBS) and the plates were incubated for 4 h at 37 °C. The formazan crystals formed were solubilized in 100 μl dimethyl sulphoxide after aspirating the medium. The extent of MTT reduction was measured spectrophotometrically at 540 nm using a Titertek Multiscan micro Elisa (Labsystems, Helsinki, Finland) and the cell survival was expressed as percentage over the vehicle. Experiments were done in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC₅₀). The IC₅₀ values were determined using GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

6. Confocal studies

Confocal microscopy is a kind of microscopy that makes use of fluorescence, the ability of a substance to give off a different wavelength of light than it absorbed. In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light as well as any fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off
some portion of the light into the detection apparatus, which in fluorescence confocal microscopy will also have a filter that selectively passes the fluorescent wavelengths while blocking the original excitation wavelength.

![Diagram of laser and detection apparatus with equation E= hv]

Figure 26. Zeiss LSM 510 META confocal microscopy and its working principle.

After passing a pinhole, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT)), transforming the light signal into an electrical one that is recorded by a computer (Figure 26). Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation [181].

Cancer cells (HeLa) were grown in a culture flask containing DMEM (Delbeco Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum) and 5% CO₂ at 37 °C. Cells were trypsinized with 0.05% trypsin–EDTA (GIBCO-25300) for 2 minutes followed by addition of 1 mL of fresh DMEM medium containing serum to inhibit trypsin activity. Cells were spun at 1200 rpm for 10 minutes and the sediment was suspended in 1xPBS (pH 7.4), placed on polylysine coated slides and incubated for one hour at room temperature for adherence. Cells were incubated with complex (40 µM) for 60 min in dark, washed twice with 1xPBS for 5 min each and then incubated with Propidium Iodide (1 µg /mL, Sigma, P4864) for 5 min followed by washing twice with 1xPBS. Cells were mounted in DABCO (Sigma, USA) and observed under EC Plan–Neofluar 40x/1.30 DIC M27 oil objective lens using ZEISS LSM–510 Meta confocal
microscope. Fluorescence emissions were recorded for compound and PI using Blue Diode 405 and HeNe 561 laser line, respectively.

7. Computational studies

The rigid molecular docking studies were performed by using HEX 6.1 software [182], which is an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein, enzymes and DNA molecule. The structure was converted to the PDB format using Mercury software (http://www.ccdc.cam.ac.uk/). The crystal structure of the B-DNA dodecamer d(CGCGAATTCGCG)2 (PDB ID: 1BNA) were downloaded from the protein data bank (http://www.rcsb.org/pdb). All calculations were carried out on an Intel pentium4, 2.4 GHz based machine running MS Windows XP SP2 as operating system. Visualization of the docked pose has been done by using CHIMERA (www.cgl.ucsf.edu/chimera) and PyMol (http://pymol.sourceforge.net/) molecular graphics program.