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DNA binding, DNA cleavage, Protein binding and Anti cancer activities of many of the synthesized metal complexes
4.1. Introduction

Cancer is one of the most leading causes of death in developed countries, responsible for about 25% of all deaths. Cancer is a disease that has a high mortality rate worldwide [1]. On a yearly basis, 0.5% of the population is diagnosed with cancer. Several approaches are employed to treat cancer, such as surgery, radiotherapy and systemic therapies like chemotherapy, endocrine therapy and targeted agents or combination of these therapies. These targeted anti-cancer therapies include monoclonal antibodies and small molecules, for example tyrosine kinase inhibitors. Conventional chemotherapeutical agents act by creating toxic effects on all dividing cells. This frequently results in severe damage of normal tissues leading to side effects like myelosuppression, alopecia, and gastrointestinal problems. The optimum goal is to find a treatment modality that specifically kills malignant cells and causes little or no side effects. The choice of the appropriate treatment depends on the nature of the tumor, the stage of the disease and the general state of the patient. The curing rate of chemotherapy has improved over the last decades, since new anti-cancer agents have been discovered, and significant advances in the different treatment protocols have been achieved [2]. Nowadays, cancer is almost always treated by a combination of several drugs (and/or applying different types of therapy).

Why to study only Lung cancer??????????

![Graph showing the 20 most commonly diagnosed cancers worldwide](image)

**Fig. 4.1.** The 20 most commonly diagnosed cancers worldwide
Fig. 4.2. The 20 most common causes of death from cancer worldwide

These reports (Figs. 4.1 & 4.2) describe the number of new cases and number of deaths due to cancer worldwide. The data are derived from the International Agency for Research on Cancer GLOBOCAN 2008 database (version 1.2), the World Health Organisation (WHO) Global Health Observatory and the United Nations World Population Prospects report [3-6].

China and India are by far the most populated countries in the world, accounting for 20% and 18% of the world’s total population in 2008, respectively [4]. Between 2003 and 2008, approximately a one third (32%) of the world’s population growth of around 400 million people occurred in India and China. India is expected to overtake China to become the world’s most populated country by 2030. The UK accounted for less than 1% of the world’s total population in 2008 [6].

The world population is ageing [6]. Between 1970 and 2010, the median age increased from 22 years to 29 years, and it is projected to reach 38 years by 2050. The number of people in the world aged 60 and over is expected to almost triple to 2 billion by 2050 [6] Since cancer is predominantly a disease of the elderly,
increases in the number of older people will inevitably lead to more cases of cancer, even if current incidence rates remain the same.

Lung cancer has been estimated as the most common cancer in the world for several decades (Figs. 4.1 & 4.2) [4, 7-11]. An estimated 1.61 million people across the world were diagnosed with lung cancer in 2008, accounting for 13% of the total [4]. More than half (55%) of the cases occurred in the developing world [4].

The link between tobacco and lung cancer was established more than fifty years ago [10] and incidence rates closely reflect past smoking prevalence with a time lag of approximately 20-30 years [4, 13, 14]. Manufactured cigarettes were first introduced at the end of the nineteenth century and since then the global consumption of tobacco has been rising steadily [14, 15]. There is estimated to be more than one billion smokers in the world, which is about a quarter of all adults. Smoking prevalence is higher in men than in women in most countries worldwide; the overall world estimates for 2006 were 41% of men and 9% of women smoking, though this varied considerably by country and age. In many developing countries the consumption of cigarettes is increasing rapidly in both sexes, due to both population growth and the increased targeting of tobacco marketing in these areas (especially to young people). Throughout most of Europe, smoking prevalence has now peaked among men but is increasing in women (particularly in the younger age groups, where teenage girls can be as likely to smoke as teenage boys). Worldwide, the number of smokers is continuing to rise, and without intervention this will lead to large increases in the incidence of lung cancer in the coming decades [14, 15].

*Why to study the interaction with DNA first??????*

DNA is a primary target for the discovery of anti-cancer drugs. Transcription and replication are vital to cell survival and proliferation as well as for smooth functioning of all body processes. DNA starts transcribing or replicating only when it receives a signal, which is often in the form of a regulatory protein binding to a particular region of the DNA. Thus, if the binding specificity and strength of this regulatory protein can be mimicked by a small molecule, then DNA function can be
artificially modulated, inhibited or activated by binding this molecule instead of the protein. Thus, this synthetic/natural small molecule can act as a drug when activation or inhibition of DNA function is required to cure or control a disease. DNA is the nucleic acid which contains the genetic 'blueprint' of all cellular life forms and of many viruses. It is responsible for two major roles: conducting its own replication during cell division and directing the transcription of complementary molecules of RNA.

DNA activation would produce more quantities of the required protein, or could induce DNA replication; depending on which site the drug is targeted. DNA inhibition would restrict protein synthesis or replication and could induce cell death. Though both these actions are possible, mostly DNA is targeted in an inhibitory mode, to destroy cells for antitumor and antibiotic action.

4.1.1. A structural introduction to DNA

Before embarking on our discussion of the binding and recognition of DNA, a brief description of the structure of DNA may be helpful.

DNA is a polymer of individual deoxyribonucleotides, each of which is composed of a heterocyclic base, a ribose sugar and a phosphate (Fig. 4.3). The most common form of DNA (and the form addressed almost exclusively in these pages) is the double-stranded, anti-parallel, right-handed double helix termed B-DNA [16, 17]. Within the polynucleotide assembly, the heterocyclic bases – adenine (A), guanine (G), cytosine (C), and thymine (T) – are bound to the sugars in an anti orientation with a disposition perpendicular to the helical axis. The base pairs collectively form a central \( \pi \)-stack that runs parallel to the helical axis between the two strands of the sugar-phosphate backbone. Each base forms hydrogen bonds with its complement on the opposite, anti-parallel strand, adenine with thymine and cytosine with guanine. The rise per base is 3.4 Å and there are ten base pairs per helical turn. Surrounding the central base stack, the polyanionic sugarphosphate backbone forms two distinct grooves, a wide major groove and a narrow minor groove (Fig. 4.3). All of these structural characteristics can and have been exploited for molecular recognition.
DNA, deoxyribonucleic acid, is the hereditary material in humans and almost all other organisms. In a person’s body each cell has the same DNA. Most of the DNA molecules located in the cell nucleus are called DNA but a small amount of DNA can be found in the mitochondria as mtDNA. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Human DNA consists of about 3 billion bases. More than 99 percent of these bases are the same in all people. The most widely accepted model for the structure of DNA molecule was proposed by Watson and Crick in 1953 for which he was awarded Nobel Prize for Medicine in 1962. According to him the DNA molecule is a double helix (Fig. 4.3). The molecule is formed by two antiparallel polynucleotide strands which are spirally coiled round each other in a right handed helix. The two strands are held together by hydrogen bonds. The double stranded helical molecule has alternated major and minor grooves. Each strand is a long polynucleotide of deoxyribonucleotides. Two strands are complementary to each other with regards to the arrangement of the bases in the two strands. Thus, in a double helix, purines and pyrimidines exist in base pairs, i.e., (A and T) and (G and C). As a result, if the base sequence of one strand of DNA is known, the base sequence of its complementary strand can be easily deduced. The backbone of the strand is formed by alternately arranged deoxyribose sugar and phosohate molecules which are joined by the phosphodiester linkages.

The DNA molecule that Watson and Crick described was in B-form. However DNA can exist in other forms also. A and B forms have right handed helix, while Z form has left handed helix. B is the major form that is found in the cell.

The following Table 4.1 summarises the different forms of DNA and Fig. 4.4 shows the different forms of DNA.

**Table 4.1. Some features of DNA forms**

<table>
<thead>
<tr>
<th>Geometry attribute</th>
<th>A-form</th>
<th>B-form</th>
<th>C-form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix sense</td>
<td>Right handed</td>
<td>Right handed</td>
<td>Left handed</td>
</tr>
<tr>
<td>Repeating unit</td>
<td>1 bp</td>
<td>1 bp</td>
<td>2 bp</td>
</tr>
<tr>
<td>Bp/tum</td>
<td>11</td>
<td>10.5</td>
<td>12</td>
</tr>
<tr>
<td>Pitch/turn of helix</td>
<td>28.2 Å (2.82 nm)</td>
<td>33.2 Å (3.32 nm)</td>
<td>45.6 Å (4.56 nm)</td>
</tr>
<tr>
<td>Diameter</td>
<td>23 Å (2.3 nm)</td>
<td>20 Å (2.0 nm)</td>
<td>18 Å (1.8 nm)</td>
</tr>
</tbody>
</table>
Fig. 4.3. *Deoxyribonucleic Acid.* (A) Structures of the 4 natural DNA bases attached to the sugar phosphate backbone. (B) The Watson-Crick base pairs with major and minor grooves illustrated. (C) Model of double-stranded, B-form DNA. The major and minor grooves are indicated. Carbon, oxygen, nitrogen, and phosphorus atoms are grey, red, blue, and orange, respectively [17]

Fig. 4.4. Different forms of DNA [17]
4.1.2. Binding to DNA

Because of the features of their tertiary structure, the different forms of DNA do not interact in the same way with drugs. The hydrophobic part constituted by the bases is not accessible to the same extent in the three main cases of binding, depending on the size of the grooves. The interactions with the bases are favoured in the case where one of the grooves is wide enough to allow the molecule to bind to it. For each form of DNA the phosphate groups make up a polyanionic backbone on the outside surface allowing interaction with the aqueous medium. The edges of the bases have hydrogen bonding potential, which is only partially satisfied by the pairing and is therefore available for recognition by ligands [18].

4.1.3. Interest of binding drugs to DNA

DNA controls the genotype of each cell and so consists of a library where all the recipes are stored. Without it, it would be impossible for cells to replicate themselves. This is the case not only for healthy cells but also for malignant cells. One approach to cancer therapy involves the use of cytotoxic agents, which reduce the proliferative drive to the tumour [18]. Many compounds with good tumour cell-killing activity have been discovered, but relatively few have found a clinical use because of the lack of discrimination between tumour and normal tissue.

The mode of action of cell-killing drugs is believed to involve the binding to DNA inside the cell, to stop the replication process. Replication is the process wherein the DNA is read base after base, translated into a codon, which codes for a particular amino acid [19]. If one base is missing or is the wrong one, the resulting nucleic acid chain or peptide, in the protein synthesis would be different; this is called mutation [20], and may lead to the death of the cell. There are two ways for the cytotoxic agents to act: the first is to bind covalently or noncovalently to the double strand and so block the RNA pathway to the replication, or more readily to cleave the strand directly.
4.1.4. DNA Binding Modes

DNA is an important genetic material in organisms and the basis of gene expression. Small molecules can interact with DNA [21-24] through intercalative binding, groove binding and electrostatic binding/external binding (Fig. 4.5).

4.1.4.1. Intercalative binding

Intercalative binding results when small molecules or the drug intercalate into the nonpolar interior of the DNA helix. Aromatic group is stacked between the base pairs in this type of binding and this happens when ligands of an appropriate size and chemical nature fit themselves in between base pairs of DNA. The ligands suitable for intercalation are mostly polycyclic, aromatic and planar and therefore often make good nucleic acid stains. There is a current interest in designing and synthesizing DNA probes, as these molecules might function as chemotherapeutic agents.

![Diagram of DNA binding modes](image)

**Fig. 4.5.** Binding modes of small molecules with DNA [17]
4.1.4.2. Groove binding

Groove binding interactions involve direct interactions of the bound molecule with edges of base pairs in either major (G-C) or minor (A-T) grooves of the nucleic acids. The antibiotic netropsin is a model groove binder in which methyl groups prevents intercalation [25]. Binding within the major groove of the double helix is rare for small molecules.

4.1.4.3. External binding

Electrostatic interaction happens in the case of positively charged molecules. They electrostatically interact with the negatively charged phosphates backbone of DNA chain. Electrostatic attraction is generally weak under physiological conditions. Cations such as Mg$^{2+}$ usually interact in this way [26].

The two most common binding modes are intercalation into the base pair stack at the core of the double helix and insertion into the minor groove. Intercalation is typically observed for cationic molecules having planar aromatic rings. The positive charge need not to be part of the ring system, but rather could be on a substituent. This binding mode requires two adjacent base pairs to separate from one another to create a binding pocket for the ligand [27]. Minor groove binders, on the other hand, usually have at least limited flexibility since this allows the molecule to adjust its structure to follow the groove as it twists around the central axis of the helix [28, 29].

![Diagram showing distortion in double helix after intercalation](image)

**Fig. 4.6.** Distortion in double helix after intercalation
Binding in the minor groove requires substantially less distortion of the DNA compared with intercalative binding (Fig. 4.6). The commonly used methods to provide insight into the binding modes of small molecules are UV-Visible spectroscopy, fluorescence spectroscopy, viscosity measurements, circular dichroism and linear dichroism.

4.1.5. Drugs which bind to DNA

Ethidium bromide is probably the best known intercalating agent [30]. Its planar aromatic unit is able to slide between the base pairs and form a strong complex (Fig. 4.7). Consequently the replication of the DNA is impossible and it is lethal for the cell [31]. A fluorimetric method for determining DNA and RNA concentrations using the fluorescence enhancement of ethidium bromide on binding to double strand nucleic-acids was proposed by LePecq and Paoletti in 1966 [32].

In the same family of intercalating drugs are acridine [33] and proflavine [34] (Fig. 4.7). They possess not only charged planar heteroaromatic rings but also amino groups able to form hydrogen bonds to acceptor sites on the DNA. Not all of the acridines are intercalative agents. The conditions of temperature and concentration can also affect the binding. At high concentration, the acridine dyes saturate the intercalative sites, after which they bind on the backbone surface [35].

The synthetic dye HOECHST 33258 (Fig. 4.7) is widely used as a fluorescent cytological stain for DNA, because it binds strongly to AT-rich sequences from the minor groove [36]. This binding is stabilized by Van der Waals interactions between the two benzimidazole rings and the sugar (04'), and hydrogen bonds between benzimidazole NH groups and proximal Adenine (N3) and Thymine (02). This groove binder is base-pair selective, by specific hydrogen bonding with the bases in the bottom of the groove and it is particularly selective for AT pairs in DNA.

As an example of these kinds of groove binders, Dervan et al. developed analogues of netropsin. The parent molecule contains a succession of pyrrole and imidazole rings interacting with the bases by specific hydrogen bonds, which targets specifically a sequence by formation of a third helix sitting in the minor groove [37].
These polyamides, because they have a higher affinity and specificity than naturally occurring DNA binding proteins, have the potential to control gene expression [38].

Actinomycin D is an intercalative antitumour drug which has also attached amino-acid side chains. It acts as a groove binder [39]. The peptide chains reside in the DNA groove, while the heterocyclic unit stacks between the base pairs. This is an example of an intercalator appended with a groove binding tail.

![Chemical structures]

**Fig. 4.7. Small molecules interacting with DNA**

4.1.6. DNA Cleavage

DNA cleavage by metal complexes generally proceeds via two major pathways by oxidative pathway and hydrolytic pathway. The DNA cleavage activity of metal complexes can be targeted towards different constituents of DNA: the eterocyclic bases, deoxyribose sugar moiety and phosphodiester linkage. Oxidative cleavage of DNA takes place in the presence of additives or photo-induced DNA cleavage. Photo-cleavers requires the presence of a photo-sensitizer that can be activated on irradiation with UV or visible light.
Many metal complexes have been studied to understand their capability in the hydrolytic cleavage of DNA which involves hydrolysis of phosphodiester bond. Nucleophilic activation is required for hydrolytic cleavage of phosphodiester bond due to unusual stability of the diester bond in DNA. Among several types of DNA cleavage reactions, those occurring under photoactivation are of particular importance in highly targeted chemotherapeutic applications. The reagents showing photo induced DNA cleavage have major advantage over chemical nucleases, as the later requires a reducing agent and/or \( \text{H}_2\text{O}_2 \) for its activity.

4.1.7. Binding with Serum Albumins

Serum albumins, as the most abundant proteins in the circulatory system, act as transporter and disposer of many endogenous and exogenous compounds [40, 41]. The crystal structure analyses of human serum albumin (HSA) have revealed that the drug binding sites are located in subdomains IIA and IIIA [42]. A large hydrophobic cavity is present in IIA subdomain. Bovine serum albumin (BSA) is structurally homologous to human serum albumin (HSA) [43]. HSA has one tryptophan (Trp-214) in subdomain IIA, whereas BSA has two tryptophan moieties (Trp-134 and Trp-213) located in subdomains IB and IIA, respectively [44, 45]. The affinities of drugs to protein would directly influence the concentration of drugs in the blood and in the binding sites and the duration of the effectual drugs and consequently contribute to their magnitude of biological actions \textit{in vivo}. Generally, the weak binding leads to a shorter lifetime or poor distribution, while strong binding decreases the concentration of free drug in plasma. Because of these, studies on this aspect can provide information on the structural feature that determines the therapeutic effectiveness of drugs and standardized screens for protein binding in new drug design and for fixing dose limits [46, 47]. Therefore, the binding of drugs to serum albumin \textit{in vitro}, considered as a model in protein chemistry to study the binding behavior of proteins, has been an interesting research field in chemistry, life sciences, and clinical medicine [48].
4.1.8. Anti-cancer studies

4.1.8.1. Cell viability (MTT) assay

The MTT Cell Proliferation and Viability Assay is a safe, sensitive, in vitro assay for the measurement of cell proliferation or when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. Cells are cultured in flat-bottomed, 96-well tissue culture plates. The cells are treated as per experimental design and incubation times are optimized for each cell type and system. The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is added to the wells and the cells are incubated. MTT is reduced by metabolically active cells to insoluble purple formazan (Fig. 4.8) dye crystals. Detergent is then added to the wells, solubilizing the crystals so the absorbance can be read using a spectrophotometer. Samples are read directly in the wells. The optimal wavelength for absorbance is 570 nm, but any filter that absorbs between 550 and 600 nm may be used. The data is analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. These assays measure cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells (cell proliferation). Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. Using a spectrophotometric method to measure the absorption of the resulting colored solution, cell proliferation (and even cell viability) can now be accurately determined.
Even the slightest color changes that occur can be determined using the yellow tetrazole MTT – the process is thus called MTT assay.

Fig. 4.8. A microtiter plate after an MTT assay. Increasing amounts of cells resulted in increased purple colouring [48]

4.1.8.1.1. Significance of MTT Assay

Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. Therefore, reduction of MTT and other tetrazolium dyes increases with cellular metabolic activity due to elevated NAD(P)H flux. Resting cells such as thymocytes and splenocytes that are viable but metabolically quiet reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction. It is important to keep in mind that assay conditions can alter metabolic activity and thus tetrazolium dye reduction without affecting cell viability and that different tetrazolium dyes will give different results depending on whether they are reduced intracellularly (MTT).

The use of the MTT assay for many different applications is well documented. Because the MTT assay can accurately determine the cell count and cell proliferation it can be used for such applications as determining the cytotoxicity of various medicinal and toxic materials since these materials to be tested would either inhibit or stimulate cell growth and propagation.

MTT assays are far more superior to the Trypan blue staining method (which is less sensitive) and much safer than radiometric methods (which uses radio active materials). MTT assay makes use of a simple process and equipment that are already in most laboratories. The MTT assay is run using a 96-well plate. A microtitre plate
reader is then used to do the reading MTT assay are readily available in kits for research purposes only. MTT assay kits are stable and can be kept in a dark refrigerated storage for up to 18 months.

4.1.8.2. LDH Release Assay

This assay is based on measurement of cytoplasmic enzyme activities released by damaged cells. Several enzyme-release assays have been described (e.g., for alkaline and acid phosphatase); however, many of these assays are hampered by (i) the low amount of endogenous enzyme present in many types of cells, and (ii) the elaborate kinetic assays required to quantitate the enzyme activities. In contrast, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is present in all cells. LDH is rapidly released into the cell-culture supernatant when the plasma membrane is damaged and is simple to assay.

Cell death can occur either by apoptosis, a highly regulated pathway involving signal transduction cascades, or by necrosis. Necrosis is accompanied by mitochondrial swelling and increased plasma membrane permeability, while apoptosis involves an articulated breakdown of the cell into membrane-bound apoptotic bodies [49]. There are a number of screening techniques available that detect cytotoxicity and cell death, independent of mechanism. Most of these assays evaluate cell viability by measuring plasma membrane permeability [50].

Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium upon cell damage or lysis, a process that occur during both apoptosis and necrosis. LDH activity in the culture medium can, therefore, be used as an indicator of cell membrane integrity and thus a measurement of cytotoxicity. Since the activity of intracellular LDH corresponds to the number of cells in the culture, quantification of LDH in cell lysates can be used as a measurement of cell growth [50, 51].

4.1.8.3. Intracellular ROS generation (DCFDA staining)

CM-H$_2$DCFDA (chloromethyl-2',7'-dichlorofluorescein diacetate) is widely used to measure oxidative stress in cells. CM-H$_2$DCFDA is resistant to
oxidation, but when taken up by cells, is de-acetylated by intracellular esterases to form the more hydrophilic nonfluorescent reduced dye dichlorofluorescin DCFH, which then is rapidly oxidized to form a two-electron oxidation product, the highly fluorescent DCF in a reaction with the oxidizing species (H₂O₂).

Reactive oxygen species (ROS) assay uses the cell permeant reagent 2',7'-dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell. After diffusion in to the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively.

The two major sources of cellular ROS are complex-I (NADH dehydrogenase ubiquinone-ubiquinol reductase) and complex-III (ubiquinol cytochrome c reductase), both part of the mitochondrial electron transport chain. These two complexes generate ROS particularly when electron transport is slowed by high mitochondrial membrane potential (Δψm). The major product of ROS in mitochondrial is in the form of superoxide and hydroperoxyl radical. Superoxide generated in complex-III occurs in the presence of slow electron transport which allows for the ubisemiquinone anion radical to react with oxygen dissolved in the membrane.

The exact source of superoxide generated by complex-I is less known and it is believed to be due to electron leakage from its iron-sulfur clusters. Low levels (or optimum levels) of ROS play an important role in signaling pathways. However when ROS production increases and overwhems the cellular antioxidant capacity, it can induce macromolecular damage (by reacting with DNA, proteins and lipids) and disrupt thiol redox circuits. In the first instance, damage can lead to apoptosis or necrosis. Disruption of thiol redox circuits can lead to aberrant cell signaling and dysfunctional redox control.

4.1.8.4. Mitochondrial Membrane Potential

The Mitochondrial membrane potential detection assay uses a unique fluorescent cationic dye, JC-1 (5, 5', 6, 6'-tetrachlorol, 1', 3, 3'-
tetraethylbenzimidazolylcarbocyanine iodide), to signal the loss of mitochondrial membrane potential. In healthy non-apoptotic cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates.

When the critical concentration is exceeded, JC1-aggregates form and become fluorescent red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence.

The aggregate red form has absorption/emission maxima of 585/590 nm. The green monomeric form has absorption/emission maxima of 510/527 nm. The JC-1 monomers and aggregates give strong positive signals, capable of yielding both qualitative and quantitative results. Detection methods include flow cytometry, fluorescence microscopy, and a fluorescent 96-well plate reader format.

4.1.8.5. Nuclear morphology assay (DAPI staining)

DAPI binds to the AT rich regions of DNA and is used to distinguish the compact chromatin of apoptotic nuclei from that of normal cells.

4.1.8.6. AO/EB staining

Acridine Orange stains live cells (green) whereas, Ethidium Bromide stains dead cells (red-to-orange) and hence this method of dual staining enables rapid and easy recognition/differentiation of live-dead cells when visualized under a fluorescence microscope [52].

4.1.8.7. Cell cycle analysis & FITC Annexin-V/PI Staining

Apoptosis is a common form of cell death in eukaryotes, playing a fundamental role during embryogenesis, in the homeostatic control of tissue integrity, tumor regression and immune response development [53]. On receiving specific
signals, a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as caspases, and perhaps other proteases, are activated in the early stages of apoptosis. These proteins cleave key cellular substrates that are necessary for normal cellular function, including structural proteins in the cytoskeleton and nuclear proteins. The caspases can also activate other degradation enzymes such as DNases, which begin to cleave the DNA at the linker regions between oligonucleosomes [54]. The result of these biochemical events is the appearance of morphological changes in the cell and extensive DNA cleavage. The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiples of 180 bp), which generate a characteristic ‘ladder’ pattern during agarose gel electrophoresis [55]. Because the DNA in apoptotic cells is partially degraded, the fraction of low-molecular-weight DNA can be extracted, whereas the non-degraded DNA remains in the cell nucleus [56]. Because DNA fragments are lost from apoptotic nuclei and nuclear DNA content can be easily measured by flow cytometry, after nucleic acid staining with specific fluorochromes [57], methods have been developed for a quantitative evaluation of apoptotic nuclei.

Basically, the method we describe uses PI for nuclear staining. PI is a fluorogenic compound that binds stoichiometrically to nucleic acids [58-60] so that fluorescence emission is proportional to the DNA (and RNA, which has to be removed if DNA is to be measured) content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels.

This method appears to offer a number of advantages. It allows (i) a rapid, reliable and reproducible estimate of apoptosis, (ii) simultaneous analysis of cell-cycle parameters of surviving cells and (iii) when necessary, simultaneous analysis of cell surface antigens recognized by fluorescein isothiocyanate- or Alexa 488-conjugated monoclonal antibodies and the extent of apoptosis [61].

It should be stressed, however, that many types of apoptosis exist, and the extensive DNA fragmentation and loss of DNA fragments is not a universal finding in apoptotic death. Also, necrotic cells sometimes display some degrees of DNA
degradation that may result in hypodiploid nuclei. Furthermore, the ‘sub-G1’ peak can represent, in addition to apoptotic cells, nuclear fragments, clumps of chromosomes, micronuclei or nuclei with normal DNA content but different chromatin structure and diminished accessibility of fluorochrome to DNA (i.e., cells undergoing differentiation).

DNA peak is not a bona fide proof of apoptotic death. Morphological (microscopic observation of apoptotic bodies) [62], biochemical (DNA ladder in agarose gel) [55] or specific demonstration of DNA breaks (terminal deoxynucleotidyl transferase assay [63]) should be used to confirm apoptosis before quantitative analysis by flow cytometry.

Another important concern in quantitative evaluation of apoptotic cells by flow cytometry is the discrimination of true apoptotic nuclei from nuclear debris. A proper setting of acquisition parameters (volume of particles, usually measured as forward scatter (FSC)) and of diploid DNA peak by using a calibration standard (DNA check beads) and negative and positive cell controls is essential before using the method with a cell line that has never been analyzed before. It should also be remembered that apoptosis is a dynamic process and that there is a short “time window” during which apoptotic cells display their characteristic features. For this reason, different methods can produce different results depending on the time of the apoptosis process [64]. For example, in early phases of apoptosis, terminal deoxynucleotidyl transferase can be positive for DNA breaks, and the cell membrane can expose phosphatidylserine which is Annexin-V positive. However, morphological observation can be negative for apoptotic bodies and flow cytometric analysis can be negative for the sub-G1 peak, as DNA fragments are still maintained in the nucleus. Accordingly, the DNA ladder cannot be observed with agarose gel electrophoresis.

However, when used appropriately, the PI flow cytometric assay is a rapid and easily reproducible method that can be adapted for apoptosis evaluation in different kinds of cells.
4.1.9. Metal complexes against different diseases

![Image showing metal complexes against different diseases: Leishmania, Trypanosomiasis, Malaria, Cancer]

Fig. 4.9. Metal complexes against different diseases

4.2. Experimental

4.2.1. Materials

CT-DNA (Calf Thymus DNA) and BSA (Bovine Serum Albumin) were purchased from Sigma Aldrich. All the chemicals used were of AR grade. Solvents used in this study were purified following the standard procedures. Dulbecco’s Modified Eagle Medium (DMEM), Trypsin Phosphate Versene Glucose (TPVG) solution Trypsin and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt. Ltd. (Bombay, India). Fetal bovine serum (FBS) was purchased from Biosera (Ringmer, East Sussex UK) and dimethyl sulfoxide (DMSO) was purchased from the Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Rhodamine 123, 4',6-diamidino-2-phenylindole (DAPI) and 2',7'-dichlorofluorescein diacetate (CM-H₂-DCFDA) were purchased from Sigma (Delhi, India).

4.2.2. DNA binding studies

All of the experiments involving the binding of complexes with CT-DNA were carried out in double distilled water with trisodium citrate (Tris, 15 mM) and...
sodium chloride (150 mM) and adjusted to pH 7.05 with hydrochloric acid. The DMF solution of the complexes was used throughout the study. The concentration of CT-DNA per nucleotide was estimated from its known extinction coefficient at 260 nm (6600 M\(^{-1}\) cm\(^{-1}\)) [65]. Solutions of CT-DNA in tris buffer gave a ratio of UV absorbance at 260 and 280 nm (A\(_{260}/A_{280}\)) 1.8-1.9 indicating that the DNA was sufficiently free of protein. Absorption titration experiments were performed by maintaining a constant metal complex concentration, while gradually increasing the concentration of DNA (μM). While measuring the absorption spectra, an equal amount of DNA was added to both the test solution and the reference solution to eliminate the absorbance of DNA itself.

The data were then fit to eq 1 [66] to obtain intrinsic binding constant K\(_b\).

\[
[DNA]/(ε_n − ε_f) = [DNA]/(ε_b − ε_f) + 1/K_b (ε_b − ε_f) \quad \ldots (1)
\]

Where, [DNA] is the concentration of DNA in base pairs, ε\(_n\) is the extinction coefficient observed for the MLCT absorption band at the given DNA concentration, ε\(_f\) is the extinction coefficient of the complex free in solution, and ε\(_b\) is the extinction coefficient of the complex when fully bound to DNA. A plot of [DNA]/[ε\(_n\)-ε\(_f\)] versus [DNA] gave a slope 1/[ε\(_n\)-ε\(_f\)] and Y intercept equal to (1/K\(_b\))[ε\(_b\)-ε\(_f\)], respectively. The intrinsic binding constant K\(_b\) is the ratio of the slope to the intercept [66].

Competitive studies of complexes as well as ligands with ethidium bromide (EB) have been investigated with fluorescence spectroscopy in order to examine whether the compound can displace EtBr from its CT DNA–EB complex. The CT DNA–EtBr complex was prepared by adding 3.3 μM EtBr and 4.2 μM CT-DNA in buffer (150 mM NaCl and 15 mM trisodium citrate at pH 7.05). The intercalating effect of the compound with the DNA–EtBr complex was studied by adding a certain amount of a solution of the compound step by step (μM) into the solution of the DNA–EtBr complex. The influence of the addition of each compound to the DNA–EtBr complex solution has been obtained by recording the variation of fluorescence emission spectra. The emission spectra were monitored by keeping the excitation of the test compound at 545 nm and the emission was monitored in the range of 550–750 nm. The emission was observed at 610 nm.
Commonly, fluorescence quenching can be described by the following Stern-Volmer equation (eq 2) [67].

$$F_0 / F = 1 + K_{sv}[Q] = 1 + k_q \tau_0 [Q] \quad \text{(2)}$$

where $F_0$ and $F$ are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, $K_{sv}$ is the Stern-Volmer quenching constant, obtained from the slope of the plot $F_0 / F$ versus [Complex], $[Q]$ is the total concentration of quencher, $k_q$ is the bimolecular quenching constant, and $\tau_0$ is the average lifetime of protein in the absence of quencher, and its value is $10^{-8}$ s.

The apparent DNA binding constant ($K_{app}$) values of the complex was obtained from this fluorescence spectral measurement. The $K_{app}$ values were obtained from the equation:

$$K_{app} \times [\text{complex}]_{50} = K_{EB} \times [\text{EtBr}] \quad \text{(3)}$$

where $K_{app}$ is the apparent binding constant of the complex studied, $[\text{compound}]_{50}$ is the concentration of the complex at 50% quenching of DNA-bound ethidium bromide emission intensity, $K_{EB}$ is the binding constant of ethidium bromide ($K_{EB} = 1.0 \times 10^7$ M$^{-1}$), and $[\text{EtBr}]$ is the concentration of ethidium bromide (3.3 μM) [68].

For viscosity measurements, the flow time was measured with digital stopwatch using Ostwald’s viscometer at 30±0.01 °C. DNA samples were prepared in water by sonication in order to minimize complexities arising from DNA flexibility. The DNA solution (50 μM) was mixed with varied concentrations (mM) of complexes in DMF for flow time measurements. The flow time reported in the present study is an average of three readings for each sample. Data were shown as $(\eta / \eta_0)^{1/4}$ vs. [Complex]/[DNA], where $\eta_0$ is the viscosity of DNA alone and $\eta$ is the viscosity of DNA in the presence of the complex. The $\eta_0$ and $\eta$ values were calculated from the observed flow time of DNA solution and DNA+metal complex solution, respectively and these values were corrected by subtracting the flow time observed for the DMF alone.

The cleavage of DNA by the Cu(II) complexes was monitored using agarose gel electrophoresis. Reactions using 3 μM DNA in water was treated with the Cu(II) complexes and 5 μM hydrogen peroxide. A loading buffer containing
0.25% bromophenol blue, 30% glycerol was added and the electrophoresis was performed at 100 V in Tris-acetate-EDTA (TAE) buffer using 1% agarose gel containing ethidium bromide. Agarose gel electrophoresis of plasmid DNA was visualized by photographing the fluorescence of intercalated ethidium bromide under a UV illuminator at 360 nm.

4.2.3. Tryptophan Quenching Studies

Similar experimental procedure was followed for tryptophan quenching study as DNA binding studies. Quenching of the tryptophan residues of BSA was performed using complexes and ligands as quencher. To the solutions of BSA in buffer, increments of the quencher were added and the emission signals at 343 nm (excitation wavelength at 296 nm) were recorded after each addition of the quencher [66].

The data were fitted in the stern-volmer eq 2 (section 4.2.2). The stern-volmer constant was obtained from the slope of the plot $F_0/F$ versus [Compound].

4.2.4. Anticancer activity

4.2.4.1. Cell culture

Human lung carcinoma (A549) cells were obtained from National Centre for Cell Science, Pune, India and were seeded (1 x 10^5 cells/ T25 flask) and cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic solution at 37°C with 5% in water Jacketed CO₂ incubator (Thermo scientific, forma II). Cells were sub-cultured every third day by trypsinization with TPVG solution. Reagents used herein were filtered through 0.22 μ filter (Laxbro Bio-medical Aids Pvt. Ltd) prior to their use for the experiment. A549 cells were maintained for a period of 24 h in absence of presence of complexes at a cell density of 5.0 x 10⁴ cells/well in 96 well plate for MTT and LDH assay and 1 x 10⁵ cells/well in 6 well plate for LDH release assay, mitochondrial membrane potential assay, DCFDA and AO-EB staining, cell cycle analysis and Annexin V-PI staining assays.
4.2.4.2. Cell viability (MTT) assay

A549 cells (7 x 10^3 cells/well) were maintained in 96-well culture plates (Tarson India Pvt. Ltd.) for 24 h in absence and presence of complexes. At the end of incubation period 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml) was added to the wells and plates were incubated at 37 °C for 4 h. Later, culture media were discarded and wells were washed with Phosphate Buffer Saline (Hi-media, India, Pvt. Ltd.), followed by addition of 150 μl of DMSO and subsequent incubation for 30 min and absorbance was read at 540 nm in ELX800 Universal Microplate Reader [69].

4.2.4.3. Cellular integrity (LDH release) assay

A549 cells were maintained in 96 well plates for 24 h as mentioned above. Later supernatant from each well was collected and activity levels of LDH were assayed with commercially available kit (Reckon diagnostics Ltd., Mumbai, India) using Merck microlab L 300 semi-auto-analyzer and % cytotoxicity was calculated [69].

4.2.4.4. Intracellular ROS generation (DCFDA staining)

After 18 h of treatment with complexes, cells were incubated with 7.5 μM chloromethyl-2, 7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) at 37°C for 30 min. Cells were observed in a Leica DMRB fluorescent microscope [70].

4.2.4.5. Mitochondrial Membrane Potential

The changes in mitochondrial membrane potential were measured using the fluorescent cationic dye Rhodamine 123 (RHO 123). After 24 h treatment with complexes, cells were incubated with 1 μM RHO 123 for 10 min at 37 °C. The fluorescence was determined at excitation and emission wavelength of 485 and 530 nm, respectively using spectrofluorometer (Jasco FP-6300, Japan) and expressed as fluorescence intensity units (FIU) [71].

4.2.4.6. Nuclear morphology assay (DAPI staining)

Cells (5×10^4 cells/well) were plated into 6-well plate. After 80% confluence, the cells were treated with or without different concentrations of complexes at 37 °C for 24 h. Single-cell suspensions of treated cells were washed with PBS and fixed with 70% ethanol for 20 min at room temperature. Cells were washed again with PBS
and stained with DAPI (0.6 μg/mL in PBS) incubated for 5 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined under a fluorescent microscope (Leica DMRB fluorescence microscope).

4.2.4.7. AO/EB staining

A549 cells (1 x 10^5 cells/well) were maintained in 6 well plates as described earlier for 24 h. At the end of experiment period, cells were collected using TPVG solution. 1 μl of dye mixture (1 mg/ml AO and 1mg/ml EB in PBS) was mixed with 9 μl of cell suspension (0.5 x 10^6 cells/ml) on a clean microscope slide and examined and photographed under Leica DMRB fluorescence microscope. A minimum of 300 cells were counted in every sample to calculate percentage cell death.

4.2.4.8. Cell cycle analysis

Cells (1 x 10^6 cells/well) were cultured as mentioned earlier for 24 h. After incubation, the cells were washed once in ice-cold PBS and subjected to cell cycle analysis [72]. Briefly, 1 x 10^6 cells were fixed in 4.5 ml of 70% (v/v) cold ethanol for 30 min, centrifuged at 400g for 5 min. Supernatant was removed and cells were washed with 5 ml of PBS. Cells were then re-suspended in 0.5 ml of PBS and 0.5 ml of DNA extraction buffer (Mix 192 ml of 0.2 M Na_2HPO_4 with 8 ml of 0.1% Triton X-100 v/v) was added. The pH was adjusted to 7.8. Cells were incubated at room temperature for 5 min and then centrifuged at 400g for 5 min. Supernatant was discarded and cells were re-suspended in 1 ml of DNA staining solution (200 mg of PI in 10 ml of PBS + 2 mg of DNase free RNase). Cells are then incubated for at least 30 min at room temperature in the dark and the cell cycle distribution was then analyzed on a flow cytometer (BD FACS Aria III, USA) using FlowJo (Oregon, USA).

4.2.4.9. FITC Annexin-V/PI Staining

Annexin-V FITC/ Propidium iodide double staining assay was used to quantify apoptosis, according to the manufacturer’s protocol (Invitrogen, UK). After incubation, cells were harvested using TPVG solution and washed with ice-cold PBS and suspended in 100 μl of 1× binding buffer (10 mM HEPES, 140 mM NaCl, and
2.5 mM CaCl₂, pH 7.4). To this mixture, 5 µl of annexin V-FITC conjugate and 1 µl of propidium iodide solution were added to each cell suspension and incubated for 15 min at room temperature in the dark. Later, samples were analyzed on flow cytometer (BD FACSARia III, USA) using FlowJo (Oregon, USA). Double staining of cells with FITC Annexin-V and PI enables the discrimination of live cells (FITC⁻PI⁻), early apoptotic (FITC⁺PI⁻), late apoptotic (FITC⁺PI⁺) or necrotic cells (FITC⁺PI⁺).

4.2.4.10. Statistical analysis

Data was analyzed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test and results were expressed as mean ± SEM using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, California, USA.

4.3. DNA binding activities of the compounds under investigation

Because DNA is the primary pharmacological target of many antitumor compounds, the interaction between DNA and metal complexes is of paramount importance in understanding the mechanism. Thus, the mode and propensity for binding of the complex to CT-DNA were studied with the aid of different techniques.

4.3.1. Electronic absorption titration

Absorption spectral titrations are the most common method to investigate the interactions of metal complexes with DNA [73]. The spectral changes reflect the corresponding changes in DNA in its conformation and structure after the complex bound to DNA. Monitoring the changes in absorption spectra of the metal complexes upon addition of increasing amounts of DNA is one of the most widely used methods for determining overall binding constants. In the UV spectra of the complex, the intense absorption bands observed are attributed to the intraligand transition of the characteristic groups of the coordinated ligand. Any interaction between complex and DNA could perturb the intraligand-centred spectral transitions as observed in the UV spectra of solution of the complexes upon addition of DNA at different concentrations. The binding of the metal complexes to DNA helix is often
characterized through absorption spectral titration, followed by the changes in the absorbance and shift in the wavelength. Hyperchromism and hypochromism are the spectral changes typical of a metal complex association with the DNA helix [74]. The extent of the hypochromism commonly parallels the intercalative binding strength. The hypochromicity, characteristic of intercalation has been usually attributed to the interaction between the electronic states of the compound chromophores and those of the DNA bases [75], while the red shift has been associated with the decrease in the energy gap between the highest and the lowest molecular orbitals (HOMO and LUMO) after binding of the complex to DNA [76]. The hypochromicity also suggests that the complex may bind to DNA by intercalation mode, due to a strong interaction between the electronic states of the intercalating chromophore and those of the DNA bases [77]. The hyperchromism observed may be a first evidence of possible external binding to CT DNA, while the existence of planar ligands may suggest stabilization upon binding to DNA; in such case, intercalation due to $\pi \to \pi^*$ stacking interactions between the base pairs of CT DNA may not be ruled out [78]. Nevertheless, the exact mode of binding cannot be merely proposed by UV spectroscopic titration studies.

### 4.3.1.1. Absorption spectral studies of 4-acyl pyrazolone based complexes

The complexes displayed intense absorption band at 250-300 nm in the UV region. Changes in the electronic absorption spectra of complexes were measured as a function of DNA concentration. There was a significant change in the absorption profile of complex on sequential addition of DNA, indicating a good binding propensity of the complex. An increase in molar absorptivity (hyperchromism) was shown by the complexes on the addition of increasing amounts of DNA. So, the above phenomena imply that the Cu(II) complexes interact with DNA by partial intercalating mode.
Fig. 4.10. Changes in the electronic absorption spectra of complex (A) 6, (B) 8, (C) 12 and (D) 20 (10 μM) with increasing concentrations (0-100 μM) of CT-DNA (Tris, pH 7.04). The arrow shows the changes on addition of the CT-DNA.

4.3.1.2. Absorption spectral studies of thiosemicarbazone based complexes

4.3.1.2.1. Simple complexes

Upon the addition of DNA, interesting changes in the absorbencies of the MLCT transition absorption bands of the complexes were observed. The observed hypochromism for both the complexes unambiguously revealed the active participation of pyrazolone moieties in association with the DNA. However, the lack of red shift suggests that the binding mode of both the complexes was not classical intercalation. Because of the bulky structure of the complexes, the aromatic rings cannot completely intercalate. Therefore, the observed spectral changes were rationalized in terms of partial intercalation.
4.3.1.2.2. Ternary complexes

In the UV region, complexes exhibited intense absorption bands around 270 nm, which was assigned to $\pi \rightarrow \pi^*$ transition of aromatic chromophore. With increasing CT-DNA (5-100 $\mu$M), the absorption bands of complex are affected, exhibiting hyperchromism in $\pi \rightarrow \pi^*$ transition of complex. A strong hyperchromic effect in $\pi \rightarrow \pi^*$ transition was observed for complexes, suggesting that these complexes possess higher propensity for DNA binding. The absorption spectra of few of the complexes in the absence and presence of CT-DNA (at a constant concentration of complex) are shown in Fig. 4.10.

To further illustrate the DNA binding strength, the intrinsic binding constant $K_b$ was determined ($16 = 4.28 \times 10^4$, $17 = 4.64 \times 10^4$ and $20 = 2.04 \times 10^5$). The binding constant of these complexes were lower in comparison to those observed for typical classical intercalators (ethidium-DNA, $1.4 \times 10^6$ M$^{-1}$) [79]. The diminution of the intrinsic binding constants could be explained by the steric constraints imposed by the ligand framework and thus encouraging intercalation binding mode for the complexes. Our results are consistent with earlier reports on preferential binding to DNA in the Cu complexes [80, 81].

4.3.2. Ethidium Bromide Displacement Assay

In order to further investigate the interaction mode between the complexes and DNA, the fluorescence titration experiments are performed. The fluorescence titration experiments, especially the EtBr fluorescence displacement experiment, have been widely used to characterize the interaction of complexes with DNA by following the changes in fluorescence intensity of the complexes. The complexes showed no fluorescence either in DMF or in presence of DNA. So, the competitive DNA binding of complexes have been studied by monitoring changes in emission intensity of ethidium bromide (EtBr) bound to CT-DNA as a function of added complex concentration get final proof for the binding of the compounds to DNA via intercalation. Though the emission intensity of EtBr in buffer medium is quenched by the solvent molecules, [82] it is enhanced by its stacking interaction between adjacent DNA base pairs. When complexes were added to DNA pretreated with EtBr
$([\text{DNA}]/[\text{EtBr}]=1:1)$, the DNA-induced emission intensity of EtBr was decreased (Fig. 4.11 (A, B, C & D)). Addition of a second DNA binding molecule would quench the EtBr emission by either replacing the DNA-bound EtBr (if it binds to DNA more strongly than EtBr) or accepting an excited state electron from EtBr. Because the complexes has planar ligands, they efficiently compete with strong intercalators like EtBr for intercalative binding sites on DNA by replacing EtBr, which is reflected in quenching of emission intensity of DNA-bound EtBr. The titrations were carried out also for the ligands (Fig. 4.11 (E & F)). The emission intensity was decreased in all the cases but it was much lower than that of the respective complexes. Ethidium bromide (EtBr) is a weak fluorophore, but its emission intensity in the presence of DNA can be greatly enhanced because of its strong intercalation between the adjacent DNA base pairs. EtBr, a planar aromatic heterocyclic dye intercalates non-specifically into the DNA which causes it to fluoresce strongly.

EtBr (weak fluorophore) + DNA (non-fluorophore) = EtBr-DNA (strong fluorophore)

In our experiments, as depicted in Fig. 4.11, the fluorescence intensity of EtBr show a remarkable decreasing trend with the increasing concentration of the complexes, indicating that some EtBr molecules are released from EtBr-DNA complex after an exchange with the complexes which results in the fluorescence quenching of EtBr. This may be due either to the metal complex competing with EtBr for the DNA-binding sites thus displacing the EtBr (whose fluorescence is enhanced upon DNA binding) or it should be a more direct quenching interaction on the DNA itself. We assume the reduction of the emission intensity of EtBr on increasing the complex concentration could be caused due to the displacement of the DNA bound EtBr by the Cu(II) complexes. Such a quenched fluorescence behavior of EtBr bound to DNA caused by the interaction between the binuclear Cu(II) complexes and DNA is also found in other copper complexes [83].
Fig. 4.11. Emission spectra ($\lambda_{em}=610$ nm) of DNA–EtBr complex in buffer solution in the absence and presence of the increasing amount of (A) 11, (B) 13, (C) 16, (D) 20, (D) TMCPMP-TS, and (D) Phen. The arrow shows the changes of intensity upon the increasing amount of compound.
<table>
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<tr>
<th>Complex</th>
<th>Apparent binding constant $K_{\text{app}}$ M$^{-1}$</th>
<th>Stern-volmer constant $K_{\text{s}}$ M$^{-1}$</th>
<th>Bimolecular constant $K_{\text{a}}$ M$^{-1}$ s$^{-1}$</th>
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Quenching data were analyzed according to the Stern-Volmer equation for the complex as well as for the ligands. The plot of $F_0/F$ versus $[Q]$ for one of the complexes and its corresponding ligands is shown in Fig. 4.12. The values of stern volmer constants were found to be $1.3 \times 10^5$ M$^{-1}$ (20), $0.6 \times 10^5$ M$^{-1}$ (TMCPMP-TS) and $0.8 \times 10^5$ M$^{-1}$ (1, 10, Phenanthroline). The values are in good agreement with the constants observed for typical classical intercalators (ethidium–DNA, $1 \times 10^7$ M$^{-1}$). The diminution of the intrinsic binding constants could be explained by the steric constraints imposed by the ligand framework and thus encouraging a partial intercalation binding mode for the complex. The figures also show that the ratio of quenching of the intensities in all complexes is different. Our results are consistent with earlier reports on preferential binding to DNA in the Cu complexes [80, 81, 84].
Further, the apparent binding constant ($K_{app}$) values obtained for the complex using the following eq 3 (section 4.2.2) and the data are listed in Table 4.2.

![Graph showing the plot of $F_0/F$ vs concentration for complex and ligands in buffer solution on increasing amount of DNA (150 mM NaCl and 15 mM trisodium citrate at pH 7.04) ($\lambda_{em}=610$ nm)](image)

**Fig. 4.12.** Plot of $F_0/F$ vs concentration for complex and ligands in buffer solution on increasing amount of DNA (150 mM NaCl and 15 mM trisodium citrate at pH 7.04) ($\lambda_{em}=610$ nm)

### 4.3.3. Viscosity measurements

Optical photophysical probes provide necessary, albeit not sufficient, insight to support a binding model. Hydrodynamic measurements, i.e. viscosity and sedimentation that are sensitive to length changes are regarded as the least ambiguous and most critical tests to a binding model in solution in the absence of crystallographic structural data [85]. A classical intercalation model usually results in lengthening of the DNA helix as base pairs are separated to accommodate the bound ligand, leading to an increase in the DNA viscosity. In contrast, semi-intercalation of a ligand could bend or kink the DNA helix, and thus reduce its effective length and, concomitantly, its viscosity. A classical intercalation mode causes a significant increase in viscosity of DNA due to an increase in separation of base pairs at intercalation sites and hence an increase in overall DNA length [86].

The viscosity measurements were studied for the binary complexes containing 4-toluoyl pyrazolones and also for simple complexes containing thiosemicarbazone based pyrazolones.
4.3.3.1. Viscosity measurements for binary complexes containing 4-toluoyl pyrazolones

![Graph showing viscosity changes](image)

**Fig. 4.13.** Effect of increasing amounts of (A) 1, (B) 2 and (C) 3 on the relative viscosities of DNA at 30.0 ± 0.1 °C. [DNA] = 1 mM, [Complex]/[DNA] = 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, respectively

The changes in the relative viscosity of rod-like DNA in the presence of these complexes are shown in Fig. 4.13. The viscosity of DNA increases greatly with increasing concentration of complex, which is similar to that of the proven intercalator EtBr [87]. This observation suggests that the principal mode of DNA binding by complexes involve base-pair intercalation, with one ligand intercalating into the base pairs and the other ligand being left outside the helix [88]. The intercalative interaction with DNA is related to the molecular structure, as in this complex there is a little distorted plane that may lead to the weak intercalative mode. This result also parallels the pronounced emission enhancement of both complexes and is comparable with the proven classical intercalator EtBr. On the basis of the viscosity results, the complexes bind with DNA through the intercalation mode. The increased degree of viscosity, which may depend on its affinity to DNA follows the order of EtBr > 3 > 2 > 1, which is consistent with the results of Cu(II) complex proposes to be bound to DNA by intercalation [89].
4.3.3.2. Viscosity measurements for simple complexes containing pyrazolone based thiosemicarbazone

The changes in the relative viscosity of DNA in presence of increasing amount of the 16 and 17 are presented in Fig. 4.14. Ethidium bromide (EtBr), a known DNA classical intercalator, increases the relative specific viscosity of DNA double helix due to lengthening of helix axis owing to its intercalative binding mode. In presence of increasing amount of the complexes, the relative viscosity of DNA increases steadily [90]. However, this increase is rather less as compared to classical intercalators EtBr.

The increasing degree of viscosity follows the order EtBr > 16 > 17. On the basis of viscosity results, at first glance, the increase in the viscosity of DNA appears to result from the intercalative interactions but the increase is quiet less than that for potential intercalator viz; EtBr with the same concentration ranges of DNA.

Thus, we conclude that, although the complexes exhibit affinity for the intercalative site on DNA but the binding affinity to a lower extent than EtBr. These results indicate that the complexes bring conformational changes in a manner, probably due to the steric matching of the DNA grooves and the complexes resulting in some partial intercalation of the complexes within the hydrophobic DNA pockets through the aromatic rings. This aggregation of the complexes into the groove of DNA helix inducing partial intercalative interactions causes an extension of the DNA helix with concomitant increase in its viscosity. Thus, the above results cumulatively indicate that the complexes bind to DNA in the groove regions [91]. Moreover, the increase in viscosity observed for complex 16 (Fig. 4.14 A) is more pronounced as compared to complex 17 (Fig. 4.14 B), indicating that complex 16 bind more strongly with DNA than complex 17.
Fig. 4.14. Effect of increasing amounts of (A) 16 and (B) 17 on the relative viscosities of DNA at 30.0 ± 0.1 °C. [DNA] = 1 mM, [Complex]/[DNA] = 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, respectively

4.4. DNA cleavage activities of the compounds under investigation

It is well documented [92] that there are specific chemical compounds that can specifically recognize and cut DNA. The potential scope of utility of these compounds is enormous and range from the creation of synthetic restriction enzymes for use by molecular biologists to the development of chemotherapeutic agents that might be effective against a variety of diseases including cancer. More appropriately both the naturally occurring DNA cleavage agents and man-made compounds that can specifically recognize and cut DNA have been termed as chemical nucleases [93]. Since DNA cleavage is one of the important mechanisms to arrest the growth of bacteria and viruses, in the control of diseases particularly cancer, there is considerable current interest in the development of reagents suitable for cleaving DNA. Further, most often the natural products prove to be toxic to the cells and hence great efforts are directed towards the design of synthetic analogs capable of cleaving DNA in a similar manner without exhibiting the associated toxicity.

Though many workers have reported several mechanisms for action of DNA cleavage such as (a) photolytic formation of phenyl cations [94], (b) alkylation of the
purines and (c) hydrolytic cleavage of double-stranded DNA involving a phosphodiester bond [92] and abstraction of a C-50 hydrogen from thymidylate and adenylate residues [95], the mechanism of action of Cu(II) complexes on DNA cleavage is underway. In order to assess the competence of the Cu(II) complexes for DNA strand scission, DNA was incubated with both Cu(II) complexes under identical reaction conditions. The cleavage reaction was monitored by gel electrophoresis. The delivery of high concentrations of metal ion to the helix, in locally generating oxygen or hydroxide radicals, leads to an efficient DNA cleavage reaction.

The characterization of DNA recognition by transition metal complexes has been aided by the DNA cleavage chemistry that is associated with redox-active or photoactivated metal complexes. DNA cleavage is controlled by relaxation of supercoiled circular form of DNA into nicked circular form and linear form. When circular plasmid DNA is conducted by electrophoresis, the fastest migration will be observed for the supercoiled form (Form I). If one strand is cleaved, the supercoiled will relax to produce a slower-moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) will be generated that migrates in between. The cleavage studies have been carried out for few of the synthesized complexes.

4.4.1. Hydrolytic cleavage

Copper(II) complexes are able to cleave DNA through both oxidative and hydrolytic processes. The possibility of a hydrolytic mechanism for the DNA cleavage by the four complexes must be taken into account. Since complexes satisfy one of the primary criteria for catalyzing hydrolytic cleavage of DNA, i.e., coordination of the phosphate moiety of DNA to the copper(II) center of complex, its DNA cleaving ability has been investigated with various concentrations of the complexes. Interestingly, complexes are found to hydrolytically cleave DNA in the absence of any reducing agent or light. Figs. 4.15 & 4.16 show the results of the gel electrophoresis experiment carried out with supercoiled DNA in the absence and presence of complexes. Lane 1 in the figures (Figs. 4.15 & 4.16) shows the control DNA without any added complexes. In the absence of the complex, DNA remains in the supercoiled form while incubation of DNA with the complex leads to its
conversion to form II. Also in the presence of Cu-salt, no cleavage is observed (Lane 2 in Figs. 4.15 & 4.16).

![Image of gel electrophoresis]

**Fig. 4.15.** Hydrolytic cleavage of DNA induced by 16. Lane 1, untreated DNA; lane 2, DNA + 60 μM Cu-acetate; lane 3, DNA + 10 μM complex; lane 4, DNA + 20 μM complex; lane 5, DNA + 30 μM complex; lane 6, DNA + 40 μM complex; lane 7, DNA + 50 μM complex; lane 8, DNA + 60 μM complex.

Fig. 4.15 shows DNA cleavage in the presence of complex 16. It was observed that the complex can cleave DNA with increase in the concentration. As shown in Fig. 4.15, with the increase of the concentrations of the complex, the supercoiled DNA decreases and nicked form gradually increases. The results indicate that the DNA cleavage activity of the complexes is obviously complex concentration dependent. It is also observed that the distance between two forms increases with the increase in the complex concentration. Fig. 4.16 shows the cleavage of DNA in the presence of 17. It was observed that complex can cleave DNA effectively in a region of micromolar concentration, as evidenced by the disappearance of Form I and then disappearance of the Form II (lanes 3-8). The distance between two forms also increases. When the concentration of the complex reached to 50 μM, complete cleavage was observed. It is clear that the degradation of DNA is highly dependent on the concentration of the complex used.
Fig. 4.16. Hydrolytic cleavage of DNA induced by lane 1, untreated DNA; lane 2, DNA + 60 μM Cu-acetate; lane 3, DNA + 10 μM complex; lane 4, DNA + 20 μM complex; lane 5, DNA + 30 μM complex; lane 6, DNA + 40 μM complex; lane 7, DNA + 50 μM complex, lane 8, DNA + 60 μM complex.

Based on our results and literature [96], the bonds between Cu(II) and labile carboxylate and/or phenolate oxygen of amino acid Schiff base could break under experimental conditions followed by simultaneous coordination of Cu(II) to the phosphate oxygen of DNA backbone by utilizing the strong Lewis acid property of Cu in +2 oxidation state which results in the breakage of the phosphodiester bond of DNA [97].

4.4.2. Oxidative cleavage

4.4.2.1. Oxidative cleavage by binary complexes of 4-toluyl pyrazolones

The electrophoresis clearly revealed that all complexes act on DNA as there was molecular weight difference between the control and the treated DNA samples. The Cu(II) complexes in the presence of H2O2 as an oxidizing agent show high nuclease activity with DNA. As a sequel to find out whether any of these synthesized complexes would exhibit DNA cleavage activities in vitro, their effect at concentrations of 10-50 μM (lanes 4-8) was studied using DNA and the results are shown in Figs. 4.17, 4.18 & 4.19, respectively. In case of complexes 1 and 2, we found similar results, while complex 3 gives different pattern. The difference between these two bands was also observed indicating the formation of linear DNA (form III).

As shown in Fig. 4.17, with the increase of the 1 concentration, the intensity of the nicked (Form II) band was found decrease and simultaneously, the circular super coiled DNA (Form I) band was also found decrease. When the complex
Fig. 4.17. Cleavage of DNA induced by 1: Lane 1, untreated DNA; lane 2, DNA + 3 μl H₂O₂; lane 3, DNA + 50 μM complex; lane 4, DNA + 10 μM complex + 3 μl H₂O₂; lane 5, DNA + 20 μM complex + 3 μl H₂O₂; lane 6, DNA + 30 μM complex + 3 μl H₂O₂; lane 7, DNA + 40 μM complex + 3 μl H₂O₂; lane 8, DNA + 50 μM complex + 3 μl H₂O₂.

Fig. 4.18. Cleavage of DNA induced by 2: Lane 1, untreated DNA; lane 2, plasmid DNA + 3 μl H₂O₂; lane 3, DNA + 50 μM complex; lane 4, DNA + 10 μM complex + 3 μl H₂O₂; lane 5, DNA + 20 μM complex + 3 μl H₂O₂; lane 6, DNA + 30 μM complex + 3 μl H₂O₂; lane 7, DNA + 40 μM complex + 3 μl H₂O₂; lane 8, DNA + 50 μM complex + 3 μl H₂O₂.

Fig. 4.19. Cleavage of DNA induced by 3: Lane 1, untreated DNA; lane 2, plasmid DNA + 3 μl H₂O₂; lane 3, DNA + 50 μM complex; lane 4, DNA + 10 μM complex + 3 μl H₂O₂; lane 5, DNA + 20 μM complex + 3 μl H₂O₂; lane 6, DNA + 30 μM complex + 3 μl H₂O₂; lane 7, DNA + 40 μM complex + 3 μl H₂O₂.
concentration was up to 50 µM (lane 8), the circular super coiled DNA (Form I) band was extremely faint, while the nicked (Form II) band was almost disappeared. Fig. 4.18 shows that on increasing the concentration of 2, we found the result similar to 1. But the difference was, when the complex concentration was up to 50 µM (lane 8), the circular super coiled DNA (Form I) and nicked (Form II) band were disappeared completely. 3 shows different cleavage than 1 and 2. As shown in Fig. 4.19 with increase of 3 concentration, the super coiled DNA (Form I) apparently convert to nicked (Form II). These results are similar to that observed for some Cu-salen complexes as chemical nucleus [98]. It is likely the generation of hydroxyl radical and/or activated oxygen mediated by the copper complex results in DNA cleavage. Further studies are required to clarify the cleavage mechanism.

4.4.2.2. Oxidative cleavage by binary complexes of 4-toluoyl pyrazolones based thiosemicarbazone

The electrophoresis clearly revealed that both complexes act on DNA as there was molecular weight difference between the control and the treated DNA samples.

The Cu(II) complexes in the presence of H₂O₂ as an oxidizing agent show high nuclease activity with DNA. As a sequel to find out whether any of these synthesized compounds would exhibit DNA cleavage activities in vitro, their effect at concentrations of 10-50 µM (lanes 4-8) was studied using DNA and the results are shown in Figs. 4.20 & 4.21, respectively. Figures show the results of resolution of DNA on gel electrophoresis experiment carried out with DNA in the absence and presence of both complexes individually. Lane 1 in the figure shows the control DNA without any additives. However, the nature of reactive intermediates involved in DNA cleavage by the complexes is not clear. The results indicated the important role of metal in these isolated DNA cleavage reactions. Incubation of DNA with H₂O₂ (5 µM) or with complex (50µM) alone does not bring about any apparent cleavage of DNA, as seen in lanes 2 & 3. Moreover, since the complex is not able to induce any effect on double stranded DNA (Lane 3), it appears that an oxidizing agent is required for producing cleavage. The DNA cleavage of complexes alone is inactive in the presence and absence of any external agents. The results indicate the importance of the metal in the complex for observing the chemical nuclease activity.
Fig. 4.20. Cleavage of DNA induced by 16 Lane 1, untreated DNA; lane 2, DNA + 5μl H₂O₂; lane 3, DNA + 50μM complex; lane 4, DNA + 10μM complex + 5μl H₂O₂; lane 5, DNA + 20μM complex + 5μl H₂O₂; lane 6, DNA + 30μM complex + 5μl H₂O₂; lane 7, DNA + 40μM complex + 5μl H₂O₂; lane 8, DNA + 50μM complex + 5μl H₂O₂.

Fig. 4.21. Cleavage of DNA induced by 17 Lane 1, untreated DNA; lane 2, DNA + 5μl H₂O₂; lane 3, DNA + 50μM complex; lane 4, DNA + 10μM complex + 5μl H₂O₂; lane 5, DNA + 20μM complex + 5μl H₂O₂; lane 6, DNA + 30μM complex + 5μl H₂O₂; lane 7, DNA + 40μM complex + 5μl H₂O₂; lane 8, DNA + 50μM complex + 5μl H₂O₂.

A decrease in intensity of DNA was observed by varying the concentration of the complexes in presence of DNA and H₂O₂. While the complex is treated with DNA in presence of H₂O₂, the disappearance of circular form takes place (lanes 4 & 5). After the disappearance of circular form, at last super coiled form disappears (lane 6). While at 40 & 50μM concentrations, the percent DNA cleavage activity occurred as evidenced by the total disappearance of DNA (lanes 7 & 8). However, as to the optimization of conditions like substrate concentration, concentration of the DNA, temperature, period of incubation, pH, etc., more detailed experiments are required to be carried out. A decrease in intensity of DNA (lanes 4 & 5) is a reflection of the mild DNA cleavage activity of the compound. The decrease in luminescence intensity in the presence of DNA by complex employed in the present study could be due to DNA
cleavage activity besides oxidation of guanine base. Thus the mono-copper complexes are promising candidates as chemical nucleases to cleave DNA with far reaching consequences.

4.5. Protein binding studies

Protein binding studies have been carried out for most of the complexes as well as their corresponding ligands. The data are listed in Table 4.3.

The tryptophan emission quenching experiments were carried out using bovine serum albumin (BSA) in the presence of complexes and ligands to investigate their interaction with proteins. Qualitative analysis of the binding of chemical compounds to BSA is usually detected by inspecting the fluorescence spectra. Generally, the fluorescence of BSA is caused by two intrinsic characteristics of the protein, namely tryptophan and tyrosine. Changes in the emission spectra of tryptophan are common in response to protein conformational transitions, subunit associations, substrate binding, or denaturation. Therefore, the intrinsic fluorescence of BSA can provide considerable information on their structure and dynamics and is often utilized in the study of protein folding and association reactions. The emission intensity depends on the degree of exposure of the two tryptophan side chains, 134 and 212, to polar solvent and also on its proximity to specific quenching groups, such as protonated carbonyl, protonated imidazole, deprotonated e-amino groups, and tyrosinate anions. The quenching of emission intensity of BSA was observed in presence of complex because of possible changes in protein secondary structure leading to changes in tryptophan environment of BSA [64].

The interaction of BSA with complex was studied by fluorescence measurement at room temperature. A solution of BSA (5 μM) was titrated with various concentrations of the compounds (μM). The effects of one of the complexes and its respective ligands on the fluorescence emission spectrum of BSA are shown in Fig. 4.22. The addition of the compounds to the solution of BSA resulted in a significant decrease of the fluorescence intensity of BSA at 343 nm. This result suggested a definite interaction of the compounds with the BSA protein [78].
Table 4.3 (a). Protein binding parameters of the complexes

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<th>$K_q$, M$^{-1}$ s$^{-1}$</th>
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</tr>
<tr>
<td>2</td>
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</tr>
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Table 4.3 (b). Protein binding parameters of the ligands

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<th>$K_q$, M$^{-1}$ s$^{-1}$</th>
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<td>$1 \times 10^{13}$</td>
</tr>
<tr>
<td>TPTPMP-TS</td>
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<td>$0.7 \times 10^{13}$</td>
</tr>
<tr>
<td>TPMP-TS</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 4.22. Changes in the fluorescence spectra of BSA through its titration with (A) 5, (B) 10, (C) 12, (D) 20, (E) TMCPMP-TS and (F) Phen at RT. The concentration of protein is 5 μM, and complex concentration was varied from 0-20 μM; pH 7.04 and λex 296 nm.

Quenching can occur by different mechanisms, which are usually classified as dynamic quenching and static quenching; dynamic quenching refers to a process in which the fluorophore and the quencher come into contact during the transient existence of the excited state. Static quenching refers to fluorophore-quencher
complex formation in the ground state. The stern-volmer constants were calculated for this system and the values are listed in Table 4.3. These constants were calculated from the slope of the plot $F_0/F$ versus $[\text{Compound}]$. (Fig. 4.23) The $K_{sv}$ values suggest that the complexes have higher binding propensity than ligands. The bimolecular constants $K_9$ were also calculated for the complexes and ligands and are presented in Table 4.3.

![Graph showing $F_0/F$ vs. [Compound] for different complexes](image)

**Fig. 4.23.** Plot of $F_0/F$ vs concentration of BSA in buffer solution on increasing amount of complex 20 and ligands (150 mM NaCl and 15 mM trisodium citrate at pH 7.04).

### 4.6. Anti cancer studies of the synthesized complexes

Some of the complexes have been screened for their anti cancer studies.

#### 4.6.1. MTT Assay

The *in vitro* antiproliferative activity of complexes was estimated in human lung carcinoma cells (A549 cell line). The complexes were incubated with A549 cells at a wide range of concentrations (from 0 to 250 μM) that inhibits the cell growth. It is known that the ability of cells to reduce MTT (methythiazoletetrazolium) provides an indication of mitochondrial metabolic activity which, in turn, can be used as a measure of viability and/or cell number. As shown in Figs. 4.24, 4.25 & 4.26, complexes showed the antiproliferative activity in dose dependent manner, and it was highest at 150-300 μM.

Similar cytotoxicity test (MTT assay) was also executed with rat cardiomyocytes (H9C2 cells) wherein, the cells were cultured in presence of
Cu(TMCPMP)(Phen). The rationale behind choosing H9C2 cells was to assess the possible cytotoxicity of complexes against a normal cell line as sensitive as rat cardiomyocytes. It was noted that complex treated cells revealed no significant cell death as evidenced by the results obtained that was comparable to that of control as shown in Figs. The same needs further investigation to probe into the underlying mechanism. Such selective inhibition on relevant cancer cells may be potentially useful in directing screen of effective anticancer agents in clinic applications. However, the anticancer activity of complexes is possibly attributable to is ability to bring about conformational changes and cleavage of cellular DNA and their ability to bind to the cellular proteins involved in inducing cancer.

![Graph](image1)

**Fig. 4.24.** Effect of 20 exposed to A549 cells on cell viability. Results are expressed as Mean ± SEM for n = 3 (replicates)

![Graph](image2)

**Fig. 4.25.** Effect of 32 exposed to A549 cells on cell viability. Results are expressed as Mean ± SEM for n = 3 (replicates)
Fig. 4.26. Effect of complexes exposed to A549 cells on cell viability. Results are expressed as Mean ± SEM for n = 3 (replicates)

4.6.2. LDH Release Assay

Further, the cytotoxic nature of complexes (Figs. 4.27, 4.28 & 4.29) was assessed by LDH release assay. The cytoplasmic enzyme LDH released from the cells and it can be correlated with cell death. LDH enzyme leaches out of dead cells and hence quantification of its content in culture media indirectly reveals extent of cell damage [99, 100]. The amount of LDH released from the cells increases with the exposure of complexes to the cells in dose dependent manner and it was highest in cells treated with complexes in the range of 150-300 μM.

Fig. 4.27. Effect of 20 exposed to A549 cells on LDH release. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 μg/ml Cu(TMCPMP-TS)(Phen)
**Fig. 4.28.** Effect of 32 exposed to A549 cells on LDH release. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 µg/ml Cu(TPMP-BA)₂

**Fig. 4.29.** Effect of complexes exposed to A549 cells on LDH release. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 µM complexes

### 4.6.3. Measurement of Mitochondrial Membrane Potential

A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria. This mitochondrial disruption includes changes in the membrane potential and alterations to the oxidation–reduction potential of the
mitochondria. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules. The resulting equilibration of ions leads in turn to the decoupling of the respiratory chain and the release of cytochrome c into the cytosol. Rhodamine 123 is lipophilic cations used as indicator dye, which accumulates in mitochondria. The fluorescent intensity is in proportion to the energy state of the mitochondria. Treatment of A549 cells with complexes resulted in a dose dependent decrement as compared to the untreated cells (Figs. 4.30, 4.31 & 4.32). These results revealed that exposure of A549 cells to complexes inducing the drop of $\Delta \Psi_{m}$ may be a possible cause for the apoptotic process.

![Mitochondrial membrane potential graph]

**Fig. 4.30.** Effect of 20 exposed to A549 cells on Mitochondrial membrane potential. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 μM Cu(TMCPMP-TS)(Phen)
**Fig. 4.31.** Effect of 32 exposed to A549 cells on Mitochondrial membrane potential. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 μM Cu(TPMP-BA)$_2$.

**Fig. 4.32.** Effect of complexes exposed to A549 cells on Mitochondrial membrane potential. Results are expressed as Mean ± SEM for n = 3 (replicates). Where, ns p > 0.05, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to 0 μM complexes.
4.6.4. Intracellular ROS generation (DCFDA staining)

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**Fig. 4.35.** Fluorescence photomicrographs of A549 cells exposed to 20

<table>
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**Fig. 4.36.** Fluorescence photomicrographs of A549 cells exposed to 32
Intracellular ROS can trigger apoptosis via initiation of downstream signals in a cell cycle [101]. CM-H$_2$DCFDA is widely used to measure oxidative stress in cells. CM-H$_2$DCFDA is taken up by cells and de-acetylated by intracellular esterases to form the more hydrophilic nonfluorescent reduced dye dichlorofluorescin DCFH, which then is rapidly oxidized to form a two-electron oxidation product, the highly fluorescent DCF in a reaction with the oxidizing species ($H_2O_2$) [102, 103]. In the present study, complex treatment showed dose dependent increment in green fluorescence of A549 cells and there was most prominent fluorescence recorded in the range 0.150-130 μM dose (Figs. 4.35, 4.36 & 4.37) which indicates that complexes elevate intracellular oxidative stress and are instrumental in generation of intracellular ROS in a dose dependent manner.
4.6.5. AO/EB staining

Fig. 4.38. Fluorescence photomicrographs of AO/EtBr staining of A549 cells exposed to (A) control, (B & C) 5 and (D & E) 9

Acridine Orange stains live cells (green) whereas, ethidium Bromide stains dead cells (red-to-orange) and hence this method of dual staining enables rapid and easy recognition/differentiation of live-dead cells when visualized under a fluorescence microscope [104]. The present study showed a dose dependent increment in EtBr positive cells following complex treatment as compared to untreated cells. Highest dose in the range of 0.150-250 μM appeared to be the most potent in induction of cell death as evidenced by high EtBr positive and very less number of AO positive cells (Figs. 4.35, 4.36 & 4.38).

4.6.6. Nuclear morphology assay (DAPI staining)

It has been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and retraction of processes [105]. DAPI binds to the AT rich regions of DNA and used to
distinguish the compact compact chromatin of apoptotic nuclei from that of normal cells. As shown in Figs. 4.39, 4.40 & 4.41, exposure of A549 cell to complexes resulted in appearance of more number of cells with condensed nuclei compared with control cells.

**Fig. 4.39.** Florescence photomicrographs of DAPI staining of A549 cells exposed to

**Fig. 4.40.** Florescence photomicrographs of DAPI staining of A549 cells exposed to

Department of Chemistry, MSU-Baroda
**Fig. 4.41.** Fluorescence photomicrographs of DAPI staining of A549 cells exposed to (A) control, (B) 5 and (C) 9

4.6.7. Cell cycle analysis

The underlying mechanism involved in the inhibition of growth of A549 cells exposed to complexes was examined by assessment of cell cycle; a method that is based on evaluation of cellular DNA content [72]. It showed that cells having a hypodiploid DNA content (sub-G1 material) that is characteristic of apoptosis and reflects fragmented DNA as shown in Figs. 4.42, 4.43 & 4.44. The majority of control cells were in Sub G0/G1 phase around 3%, G0/G1 phase around 93%, S phase around (2%) and G2+M around (1.5) whereas, in the range of 150-250 μM complexes treated cells were increased in Sub G0/G1 and decreased in G0/G1 phase. Increased Sub G0/G1 phase suggested DNA fragmentation and the G2-M and S phases were slightly decreased after the treatment, indicating that it may induce the G0/G1 growth arrest A549 cells. These results indicate that complexes induced apoptosis is likely to involve the modulation of cell cycle progression.
Fig. 4.42. Cell cycle analysis of A549 cells exposed to 20

Fig. 4.43. Cell cycle analysis of A549 cells exposed to 32
**Fig. 4.44.** Cell cycle analysis of A549 cells exposed to complexes

### 4.6.8. FITC Annexin-V/PI Staining

Apoptosis is a process of cell death characterized by various morphological and biochemical alteration leading to cell disruption and formation of apoptotic bodies. One of the hallmarks of apoptosis is that membrane phospholipid such as phosphatidylserine rearrange from inner to outside surface of the plasma membrane and gets exposed on cell surface. The same can be detected due to its high affinity for annexin V. This assay is based on the ability of the protein annexin V to bind to phosphatidylserine (PS) exposed on the outer membrane leaflet in apoptotic cells (PS also appears on the necrotic cell surface). In viable cells, PS is located in the inner membrane leaflet, but upon induction of apoptosis it is translocated to the outer membrane leaflet and becomes available for annexin V binding. The addition of PI enabled viable (AnnV<sup>neg</sup>/PI<sup>neg</sup>), early apoptotic (AnnV<sup>neg</sup>/PI<sup>neg</sup>), late apoptotic (AnnV<sup>pos</sup>/PI<sup>pos</sup>) and necrotic (AnnV<sup>neg</sup>/PI<sup>neg</sup>) cells to be distinguished. Flow cytometric assay with Annexin V/PI double staining of control and complexes treated cells showed that apoptosis rate was increased with complex treatment wherein, the treated cells recorded higher % annexin V positive (early apoptotic) and lower % PI positive cells (late apoptotic) (Figs. 4.45 & 4.46).
Fig. 4.45. Annexin V-Alexa fluoro 488/PI staining of A549 cells exposed to 20

Fig. 4.46. Annexin V-Alexa fluoro 488/PI staining of A549 cells exposed to 32
Conclusions

One of the most rapidly developing areas of pharmaceutical research is discovery of drugs for cancer therapy. Lung cancer has been estimated as the most common cancer in the world for several decades. In this view, we have synthesized different types of Cu(II) complexes. These complexes have bio-active ligands and so we considered that these can be a series of good anti-cancer drugs. DNA is a primary target molecule for any drug discovery. In this regards, the interaction of our complexes with CT-DNA have been studied by absorption titration, ethidium bromide displacement study and viscosity measurements. It suggested that the complexes have good binding capacity with CT-DNA. Artificial nuclease activity has been ascertained by gel electrophoretic mobility assay; the complexes display efficient cleavage activity of DNA converting the Form I to Form II and ultimately leading to the formation of linearized Form III with increasing concentrations of the complex. The DNA cleavage activity of some of the complexes has been studied by both hydrolytic and oxidative pathways. We found that the complexes can cleave the DNA even in the absence of any external indicator and the cleavage. This work presents a good overall correlation between DNA binding and DNA cleavage activity of the complexes. The results are of importance towards further designing and developing Cu(II) based complexes and systematic assessment of DNA binding, cleavage activity for their potential applications as therapeutic agents. Further, the interaction of these complexes with bovine serum albumin (protein) has also been studied by tryptophan quenching assay. It suggested the higher binding of the complexes with this protein. Generally, the weak binding leads to a shorter lifetime or poor distribution, while strong binding decreases the concentration of free drug in plasma. Because of these, studies on this aspect can provide information on the structural feature that determines the therapeutic effectiveness of drugs and standardized screens for protein binding in new drug design and for fixing dose limits. The anticancer activity of the complexes on A549 (human lung cancer) cell line has been studied. Anticancer study also revealed high grade of cytotoxicity and cell cycle arrest in sub G0/G1 phase implying to the potency of complexes as an anticancer agents against A549 lung carcinoma.
cells. Results envisaged herein indicate that complexes hold sufficient merit to develop it as a therapeutic agent against cancer.

The presence of copper with a daily intake not exceeding 4-7 mg is safe and can minimize the above diseases. Therefore, it is appropriate to design anticancer drugs based on copper, which is anticipated to cause lower toxic effects. In this context, we have started to develop ternary complexes of Cu(II) that are expected to be less toxic but highly potent in smaller dosage compared to cisplatin and analogous compounds.
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