CHAPTER I

INTRODUCTION

Metals and Biology

The involvement of metal ions in biological processes and the role of metal complexes in biological systems have culminated in the emergence of a new branch viz., Bio-inorganic chemistry. Metal ions influence biological phenomena by interacting with organic functional groups on biomolecules, forming metal complexes. From this perspective, bioinorganic chemistry may be considered as coordination chemistry applied to biological questions. In general, bioinorganic chemists tackle such problems by first focusing on the elucidation of the structure of the metal complex of interest and then correlating structure with function. The attainment of solutions usually requires a combination of physical, chemical, and biological approaches. The chemical and physical properties of metals enable organism to undertake critical processes such as respiration, metabolism, nitrogen fixation, photosynthesis, development, nerve transmission, muscle contraction, signal transduction and protection against toxic and mutagenic agents (Lippard SJ, 1994). The alkali and alkaline earth metal cations such as
Na\(^{2+}\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\) have importance as structural elements, components of ion pumps in maintaining osmotic balance within cells.

Metalloenzymes many of which incorporate the transition metal cations \(\text{Fe}^{2+}, \text{Fe}^{3+}, \text{Cu}^{2+}, \text{Co}^{2+}, \text{Mn}^{2+}\) and \(\text{Zn}^{2+}\) constitute about 30% of all the known enzymes (Shriver DF, 1995). Transition metal cations participate in a diverse range of biological catalytic, transport and redox functions (Mills AL, 2002; Roat-Malone RM, 2002). The utilization of metals for particular function is based on availability, size, stereochemistry, hard-soft acid-base character or reduction potential. In particular, transition metals are exploited for their ability to form stable cations and various oxidation states and geometries, and accept electrons from donor to form coordinate covalent bonds.

Metals and their compounds play a major role in medicine, inorganic chemistry and nutrition. The precious metals, gold and copper, have been used in medicines for thousands of years by the Egyptians, Arabs, Chinese, Indians etc. (Orvig C, 1999). The importance of zinc and iron in the diet and for the promotion of wound healing has long been known. On the other hand the toxic effects of metals such as arsenic, mercury and lead have also been long recognised (Baldwin DR, 1999). It is only within the last century that rational development of inorganic drugs commenced.
The modern discipline of inorganic medicinal chemistry is said to have begun in 1960’s with the accidental discovery of the antitumour properties of cisplatin, \([\text{Pt} (\text{NH}_3)_2\text{Cl}_2]\) \((\text{Wong E, 1999; Blower PJ, 1999})\). From this fortuitous find emerged the study of platinum anticancer agents, currently one of the most published areas of inorganic drug development. \((\text{Blower PJ, 2004; Desoize B, 2002; Desoize B, 2004; Barnes KR, 2004; Haiduc I, 1990})\)

Before the discovery of cisplatin’s anticancer qualities, the toxic and potentially carcinogenic properties of certain metal-containing substances may have been a factor delaying the investigation of metals for their biological activity \((\text{Shaw CFI, 1999})\).

The gold complex auranofin has been used for many years as an anti-rheumatic. Although they share the anti-inflammatory qualities posessed by organic drugs aspirin and ibuprofen, gold drugs are additionally able to retard the arthritis and cause remission of the disease state \((\text{Eisler R, 2003})\).

**Introduction to Cancer**

Cancer embraces a multitude of diseases with different etiologies, presentations and degrees of severity. It poses an ever increasing threat to populations and health care systems on all fronts, local, national and international. It has no boundaries, affecting people across genders, ages,
ethnicities and geography. It comes under a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system). There are many types of cancer. Severity of symptoms depends on the site and character of the malignancy and whether there is metastasis. The unregulated growth that characterizes cancer is caused by damage to DNA, resulting in mutations to genes that encode for proteins controlling cell division. Many mutation events may be required to transform a normal cell into a malignant cell. These mutations can be caused by radiation, chemicals or physical agents that cause cancer, which are called carcinogens, or by certain viruses that can insert their DNA into the human genome. Mutations occur spontaneously, and may be passed down from one cell generation to the next as a result of mutations within germ lines. However, some carcinogens also appear to work through non-mutagenic pathways that affect the level of transcription of certain genes without causing genetic mutation. Many forms of cancer are associated with exposure to environmental factors such as tobacco smoke, radiation, alcohol, and certain viruses. Some risk factors can be avoided or reduced.
Carcinogenesis, which means the initiation or generation of cancer, is the process of derangement of the rate of cell division due to damage to DNA. Cancer is, ultimately, a disease of genes. In order for cells to start dividing uncontrollably, genes which regulate cell growth must be damaged. Proto-oncogenes are genes which promote cell growth and mitosis, a process of cell division, and tumor suppressor genes discourage cell growth, or temporarily halt cell division in order to carry out DNA repair. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell. Cancer pathology is ultimately due to the accumulation of DNA mutations that negatively effect expression of tumour suppressor proteins or positively effect the expression of proteins that drive the cell cycle. Substances that cause these mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens (Van Waes C, 2007). Particular substances have been linked to specific types of cancer. Tobacco smoking is associated with lung cancer. Prolonged exposure to radiation, particularly ultraviolet radiation from the sun, leads to melanoma and other skin malignancies. Breathing asbestos fibers is associated with mesothelioma. In more general terms, chemicals called mutagens and free radicals are known to cause mutations. Other types of mutations can be caused by chronic inflammation, as neutrophil
granulocytes secrete free radicals that damage DNA. Chromosomal translocations, such as the Philadelphia chromosome, are a special type of mutation that involves exchanges between different chromosomes. Many mutagens are also carcinogens, but some carcinogens are not mutagens. Examples of carcinogens that are not mutagens include alcohol and estrogen. These are thought to promote cancers through their stimulating effect on the rate of cell mitosis. Faster rates of mitosis increasingly leave fewer opportunities for repair enzymes to repair damaged DNA during DNA replication, increasing the likelihood of a genetic mistake. A mistake made during mitosis can lead to the daughter cells receiving the wrong number of chromosomes, which leads to aneuploidy and may lead to cancer.

Furthermore, many cancers originate from a viral infection; this is especially true in animals such as birds, but also in humans, as viruses are responsible for 15% of human cancers worldwide. The main viruses associated with human cancers are human papilloma virus, hepatitis B virus, Epstein-Barr virus, and human T-lymphotropic virus (Vile RG, 2006, 2007). It is impossible to tell the initial cause for any specific cancer. However, with the help of molecular biological techniques, it is possible to characterize the mutations or chromosomal aberrations within a tumor, and rapid progress is
being made in the field of predicting prognosis based on the spectrum of mutations in some cases.

Malignant tumor cells have distinct properties:

- evading apoptosis
- immortalization due to overabundance of telomerase
- self-sufficiency of growth factors
- insensitivity to anti-growth factors
- increased cell division rate
- altered ability to differentiate
- no ability for contact inhibition
- ability to invade neighbouring tissues
- ability to build metastases at distant sites
- ability to promote blood vessel growth (angiogenesis)
### Characteristics of normal and cancerous cells

<table>
<thead>
<tr>
<th>NORMAL</th>
<th>CANCER</th>
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<tr>
<td>Large number of dividing cells</td>
<td>Large, variable shaped nuclei</td>
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<td>Small cytoplasmic volume relative to nuclei</td>
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<td></td>
<td>Variation in cell size and shape</td>
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<td>Loss of normal specialized cell features</td>
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<td>Disorganized arrangement of cells</td>
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<td>Poorly defined tumor boundary</td>
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**Normal Cell Division**
- cell damage — no repair
- apoptosis

**Cancer Cell Division**
Cancer develops from cells that are capable of dividing. All tissues in the body contain some cells that can divide and renew themselves. A subset of the cell population in any tissue can differentiate into the functional cells of that tissue. The normal process of cellular differentiation ultimately leads to an adult, fully differentiated, "dead-end" cell that cannot, under ordinary circumstances, divide again. These fully differentiated cells are the workhorse cells in most tissues in the body. Under circumstances that are not clearly understood, cells that have the potential to divide can be changed by interaction with carcinogenic agents into a cell type that is capable of continued proliferation and thereby is prevented from achieving the normal state of complete differentiation. The carcinogen-altered cell is said to have undergone malignant transformation. Somehow, the genes controlling cell proliferation are locked in the "on" position when they should be in the "off" position, and the genes controlling differentiation are either not expressed or are expressed only imperfectly (Nowell PC, 1986). Cancer is one of the three leading causes of death in industrialized nations. Cancers are caused by the progressive growth of the progeny of a single transformed cell. Only by understanding these mechanisms, the manner in which cells are altered during malignant transformation be ascertained. Therefore, curing cancer requires that all the malignant cells be removed or destroyed without killing the patient (Pardoll DM and Jaffee EM, 2000).
A. Programmed cell death: Apoptosis

B. Final stages of Apoptosis
An attractive way to achieve this would be to induce an immune response against the tumor that would discriminate between the cells of the tumor and their normal cell counterparts. Immunological approaches to the treatment of cancer have been attempted for over a century, with tantalizing but unsustainable results. Experiments in animals have, however, provided evidence for immune responses to tumors and have shown that T-cells are a critical mediator of tumor immunity. More recently, advances in our understanding of antigen presentation and the molecules involved in T-cell activation have provided new immunotherapeutic strategies based on a better molecular understanding of the immune response (Bevan MJ, 1995). These are showing some success in animal models and are now being tested in human patients.

Treatment of Cancer

The diagnosis of cancer in the earlier stages is very difficult since there are many factors that different from patient to patients. The important once are patients’ immunologic functions, performance status of the patients, the presence of complicating non-neoplastic diseases and the site of dominant metastases in patients with more advanced tumors.
Tumor is a form of tissue growth which was conventionally been divided into Benign and Malignant. Benign tumors grow slowly and do not harm the surroundings whereas the malignant tumors grow rapidly and invade surrounding tissues which lead to metastases. Antigens from such tumors react with embryonic tissue and may lost in the process of normal cell differentiation and those which escape or persist may finally lead to the development of cancer in later life.

At present there are number of methods for the treatment of cancer. They are surgery, radiation, chemotherapy and immunotherapy. The aim of these treatments is to increase the quality and duration of patients’ life. In some cases integrated cancer treatment i.e. a combination of two or more of the above methods helps to attain a better recovery with in a short period.

Metallodrugs and Cancer

Cancer is a malignant disease in which rapid and uncontrolled proliferation of cells occurs. Defective cellular apoptosis (programmed cell death) is known to be significant to the development of cancer (Kasibhatla S, 2003). Therefore proper strategy when treating cancer is to attempt to induce apoptosis. Damage to DNA, the prime target of anticancer chemotherapy, triggers processes which lead to the prevention of cellular
reproduction, and death of the cell (Kahlem P, 2004). This cell death may occur as a result of apoptosis, or premature senescence due to the cell cycle being permanently halted. A drug can interact with DNA by a number of modes in order to exert a negative effect, including alkylation, intercalation, groove binding and coordination to bases. (Frausto da Silva JJR, 1994). The drugs exerting an effect on mitochondria play an important role in energy production, molecular metabolism and regulation of apoptosis (Pretson TJ, 2001). Delocalised lipophilic cations are ideal drug candidates for targeting the mitochondria. They selectively accumulate within the mitochondria due to the high negative membrane potential and they are able to pass through the hydrophobic lipid layer due to the positive charge being delocalised over a large surface area (Modica-Napolitano JS, 2001). The difference in membrane potential between tumour cells and control epithelial cells has been found to be sufficient for ten-fold greater accumulation of lipophilic cations in cancerous mitochondria (Modica-Napolitano JS, 2001).

Traditionally, drugs used for cancer chemotherapy are organic compounds, either synthetic or natural products and include alkylating agents, antibiotics, alkaloids enzymes and hormones. There are now a large number of metal containing compounds with antitumour properties in clinical use and are at varying stages of evaluation.
Transition Metal Complexes in Cancer

The essential role of transition metal ions in biological systems is well known. When designing metal complexes for therapeutic use the following events need to be considered: hydrolysis-protein binding-membrane transport-molecular target. Hydrolysis of metal complexes is important because of the aqueous milieu of biological systems, but the hydrophobic nature of cell membranes, vesicles and enzyme active sites requires consideration of lipophilic ligands in the design of complexes. Therefore, design of metallo-drugs requires bringing together organometallic chemistry with traditional aqueous coordination chemistry, a merger that is in its infancy. Whether or not the ultimate target of a metallo-drug is a protein, protein binding is always a factor in the medicinal use of such compounds. The greatest hurdle, however, is transport of metal complexes through cell membranes, which determines if metals enter cells with their ligands intact.

Cisplatin is the most widely used antitumor drug, especially for the treatment of testicular and ovarian cancers. However, cisplatin has some major drawbacks: severe toxic side effects, a limited applicability to a relatively small range of tumors, and often occurring resistance alongwith kidney and gastrointestinal problems, including nausea, which may be attributed to the inhibition of enzymes through coordination of the heavy
metal platinum to sulfhydryl groups in proteins. Accordingly a treatment with these metal chelates may counteract these symptoms. The second generation platinum compound, carboplatin, is used clinically worldwide, and two more cisplatin analogues, nedaplatin and oxaliplatin are also used clinically. (Barnes KR, 2004). Like cisplatin, all of these compounds are platinum (II) square planar complexes in which the ligands are arranged in a cis configuration, hence the similarities observed in their chemical properties and the mechanism of action (Desoize B, 2004). In search for other antitumor active metal complexes, several ruthenium complexes have also been reported to be promising as anticancer drugs.

**Coordination Compounds as Anti-Tumour Agents**

Coordination compounds have been found have application in the field of medicine as antiviral, antibacterial and antituberculosis drugs. The importance of metal complexes in cancer treatment started when Rosenberg noted the activity of cis – dichlorodiamine Platinum (II) against cancer. The approval of the same drug for human treatment in 1979 aroused the interest of coordination chemist towards cancer.

Currently one of the most widely used cancer drugs; cisplatin is effective for the treatment of testicular, breast, ovarian, colorectal, lung,
cervical, bladder, head and neck cancers. (Haiduc I, 1990; Guo Z, 1999; Reedijk, 1996). Cisplatin, Pt(NH$_3$)$_2$Cl$_2$, is a square planar platinum(II) complex containing two amines, which are inert to substitution, and two chloride ligands, which serve as good leaving groups. While Pt (NH$_3$)$_2$Cl$_2$ can exist in both cis- and trans- forms, only the cis- configuration is effective. The trans isomer has no significant bioactivity. Over the past thirty years, major efforts have been directed towards investigating the way cisplatin exerts its effect on cells, in order to understand the reasons for its pharmacological and toxic properties, and to provide direction for the development of new drugs. The cytotoxicity of cisplatin can be attributed to the ability of the drug to modify the structure of DNA so that it is able to avoid excision repair by enzymes (Berners-Price SJ, 1996). The three major limitations to the use of platinum (II) cisplatin analogues as anticancer treatment are:

1. Severe adverse effects in patients, often resulting from lack of selectivity between tumour cells and healthy tissues.

2. The development of resistance to the drugs by tumours.

3. The restricted range of cancer that are responsive (Wong E, 1999).
Resistance to cisplatin is a growing problem which is still not properly understood. The mechanisms are extremely complex, but factors that mediate resistance may include reduced uptake and increased efflux of the drug, drug interaction with molecules other than DNA and increased DNA repair (Desoize B, 2004). Oxaliplatin demonstrates no cross resistance with cisplatin, and in addition shows good efficacy against tumours that are resistant to cisplatin. However, the side effects of oxaliplatin include significant and unpredictable sensory neurotherapy (Barnes KR, 2004). Trinuclear Pt (II) antitumour agents, BBR3464 is currently in phase II clinical trials. In preclinical trials, BBR3464 showed a lack of cross-resistance with cisplatin against various tumour lines, and was found to be 40 times more active (Perego P, 1999; Ratesi G, 1999; Orlandi L, 2001). The phase I and phase II clinical trials have demonstrated responses towards melanoma, ovarian, gastric, lung, colon and pancreatic cancers (Perego P, 1999; Pratesi G, 1999; Jodrell DI, 2004; Kasparkova J, 2004).

Antiarthritic gold (I) thiolates and phosphine gold (I) thiolates such as auranofin were tested for their anticancer activity in vitro and invivo in 1986 (Merabelli CK, 1986). The compounds exhibited activity against P388leuæmia in mice, with gold thiolates found to be less active than the phosphine gold (I) thiolates, indicating that the phosphine ligand is
important for activity. Budotitane and other diacidobis (β-ketonato) metal (IV) complexes containing Ti, Zr, Hf have shown antitumour activity against animal tumours (Keppler BK, 1991; Clarke MJ, 1999). Up to 200 variations of budotitane have been tested (Keppler BK, 1993). Variations involved the leaving groups, metal and β-ketonato ligand and it was found that asymmetric ligands are needed for high activity, with planar groups enhancing this activity. Ruthenium (II) and (III) antitumour compounds exhibit a wide variety of structural characteristics. They include octahedral complexes with amine and imine ligands, heteroaromatic ligands, DMSO Ligands. The relatively new complexes containing polyaminocarboxalate Ligands (such as PDTA = 1, 2-propylene diamine tetraacetate) and Ruthenium (II) or (III) complexes of more than one of the above ligands combined (Clarke MJ, 2003; Alessio E, 2004).

The “activation by reduction” hypothesis, put forward by Clarke et al suggests that Ru (III) drugs can be thought of as prodrugs, which are reduced to the more active R (II) species in vivo (Clarke MJ, 1999; Clarke MJ, 1993). Ruthenium complexes containing nitrogen donors which show anticancer activity include [RuCl(NH₃)₅], [cis-RuCl₂(NH₃)₄] Cl, and (HIm) [trans-RuIm₂Cl₄]. The probable reduction product of prototypical Ru(III) complexes such as [RuCl(NH₃)₅]²⁺is [Ru(H₂O)(NH₃)₅]²⁺. A mechanism by
which this species induces its effect on nucleic acid function has been described by Clarke (Clarke MJ, 1986). RAP, H [cis-RuCl₂(PDTA)] has been shown to dissociate from its chlorides in solution, while maintaining its +3 oxidation state (Gonzalez Vilchez F, 1998). The ruthenium (III) complex Him [trans-RuCl₄(DMSO-S)Im] (NAMI-A) is active against solid tumour metastases, which are usually insensitive to cisplatin (Alessio E, 2004). It is the only ruthenium complex to successfully complete a phase-I clinical trial (Bacac M, 2004).

Copper, Iron and Zinc as Antitumour Agents

Copper and iron although present in small amounts in the body, are vital for the normal functioning of several enzymes and proteins involved in energy metabolism, respiration and DNA synthesis. Their ionic forms are prone to participate in one-electron transfer reactions and can thereby catalyse redox reactions. So they generate free radicals which can induce apoptosis thereby initiating anti tumour activity. The uptake of zinc - bis (thiosemicarbazone) complexes in human cancer cells has been studied by fluorescence microscopy and the cellular distribution established, including the degree of uptake in the nucleus (Andrew R, 2005). Thiosemicarbazones and their metal complexes have been known for many years to show a broad spectrum of therapeutic properties against a range of diseases with
antibacterial, antimalarial, antiviral and antitumour activities. Despite a sustained level of interest in the pharmacological properties of such complexes, details of the cellular distribution of these complexes are scarce, particularly in living cells. Certain zinc thiosemicarbazone complexes have been shown to be active as anti-tumour agents, are as cytotoxic as cisplatin and are also effective against cisplatin resistant cell lines. (Bernado H, 2004).

N-(2-hydroxyacetophenone) glycinate (CuNG) has been synthesized, which was initially found to be a potential resistance modifying agent and later found to be an immunomodulator in mice model in different doses. Oxidative stress is linked to carcinogenesis as well as to sensitivity or resistance of cancer cells to anticancer drugs (Valko M, 2006). The involvement of reactive oxygen species (ROS) in induction of apoptosis of various cancer cells, especially drug resistant cancers is well known. Often, the ability of a therapeutic agent to induce apoptosis of cancer cells depends upon the ability of cancer cells to generate ROS.

Moreover, low levels of ROS favor the expression of ABC transporters like P-gp. drug resistant cancers often show very low levels of ROS (Park MT, 2005). This is usually due to high intracellular reduced glutathione (GSH) levels and enhanced activities of antioxidant enzymes like glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase.
(SOD) (Morales MC, 2005). On the other hand, GSH is also required for phase II detoxification reactions, for example phase II enzymes like glutathione S-transferase (GST) isozymes require GSH for the conjugation of electrophilic drugs and xenobiotics. Therefore, high levels of GSH and GST have been implicated in drug resistant tumors.

Majumder et al (Majumdar S, 2003) reported the synthesis, toxicity and resistance reversal activity of the complex, viz., copper N-(2-hydroxyacetophenone) glycinate (CuNG). The CuNG treatment alone at a lower dose (single administration) was found to completely heal EAC/Dox bearing animals from their tumor through immunomodulation in vivo. (Mookerjee A, 2006) Since copper has been reported by others to induce apoptosis by generation of ROS, and since CuNG is a copper (II) chelate, it warranted a study on its effect on ROS generation, which includes beside GSH, a number of antioxidant enzymes, like GPx, SOD and CAT.

Zhong, X (Zhong X, 2006) prepared mononuclear complexes of Zn(II), Cu(II), Mn(II), Co(II), Ni(II) with a new Schiff base ligand derived from 2,3-butanedione and thiosemicarbazide. Among these complexes, Cu (II) complex has highest antitumour activity. Giuseppe Filomini et al Giuseppe Filomini, 2007), characterized the pro-apoptotic activity of two new synthesized isatin-Schiff base copper (II) complexes, obtained from isatin and
1, 3-diaminopropane or 2-(2-aminoethyl) pyridine: (Cu(isapn)) and (Cu(isaepy)$_2$), respectively. It was demonstrated that these compounds trigger apoptosis via the mitochondrial pathway. The extent of apoptosis mirrors the kinetics of intracellular copper uptake. Particularly, Cu (isaepy)$_2$ enters the cells more efficiently and specifically damages nuclei and mitochondria, as evidenced by atomic absorption analysis of copper content and by the extent of nuclear and mitochondrial integrity.

Mohammad Hashemi et al (Heshemi, 2007) reported the cytotoxic effects of intra and extra cellular zinc chelation on human breast cancer cells. Zinc serves many essential functions in mammalian cells and its important micronutrient in structural and regulatory functions. Zinc interacts with zinc bounding motifs, so called ‘zinc finger’ domains and thereby act as a cofactor for several hundred enzymes. Zinc also specifically bind to various membrane receptors, transporters and channels thereby modulating their activity (Huang, 1997). Co (II), Ni (II), Pd (II) and Pt (II) complexes of ortho-naphthaquinone (1, 2-dione) thio semicarbazone were synthesized and characterized by spectroscopic studies (Znhra A, 2004). In vitro anticancer studies on cancer cells reveals that adding a thiocompound to a parent dione carbonyl, considerably enhances its antiproliferative activity. Carol Deegan et.al (Deegan C, 2006) reported the anticancer chemotherapeutic potential of 1,
10-Phenanthroline 5, 6-dione (Phenadione) and its Co (II) and Ag (I) complexes in human cell lines. The results of their study suggest that Phenadione and its reported complexes may be capable of acting as highly effective anticancer therapies, which with careful administration could provide very potent and effective alternatives to cisplatin. Transition metal cations such as Cu (II) and Fe (II) bind to negatively charged DNA and have been shown to play an important role in the local formation of OH radicals (Samuni A, 1981; Wijker CA, 1999).

As discussed, the behaviour of metal complexes in biological systems is dependent on the following factors (1) the identity of the metal, (2) the oxidation state of the metal (3) the degree and type of derivatisation of the aromatic ligands. Regardless of the mode of action of these various drug candidates, it is established that activity can be greatly influenced by changes to the structure of the metal complex. The structure activity relationship that is observed may then provide more clues concerning the biological targets and the way cellular death is carried out. This in turn may provide a rational strategy for much needed design and synthesis of new anticancer metallodrugs.
Scope of the Present Investigation

Many metallic elements play a crucial role in living systems. A characteristic of metals is that they easily lose electrons from the familiar elemental or metallic state to form positively charged ions which tend to be soluble in biological fluids. It is in this cationic form that metals play their role in biology. Metal ions are electron deficient, most biological molecules such as proteins and DNA are electron rich. The attraction of these opposing charges leads to a general tendency for metal ions to bind to and interact with biological molecules. Inorganic compounds have had an enormous impact on medicine, in particularly in the treatment of cancer. Recently there have been a number of reports highlighting the use of transition metal complexes as anticancer agents.

Co-ordination of a metal ion to a ligand can show large conformational changes, with concomitant bond elongations or compressions which can have drastic effects on its properties, which are most obvious in the spectroscopic and biological properties. But there are only few reports on the antitumour activities of Schiff bases derived from aminophenol, aminothiophenols, 1, 2-diketones and hydroxyketones. Our present study involves the synthesis of transition metal complexes of Schiff bases such as 2-Hydroxyacetophenone 2-Aminothiophenol (HAPATP),
2-Hydroxyacetophenone 2-Aminophenol (HAPAP), Benzil 2-Amino-thiophenol (BATP), Benzil 2-Aminophenol (BAP) and testing their cytotoxic potential in HeLa and DLA tumor cell lines.

Their ligands and complexes have characterized by analytical and spectroscopic methods which are discussed in Part I. The transition metal ions used for the complex formation are Co (II), Ni (II), Cu (II) and Zn (II). The specific objectives of the present study were; 1) Determination of *in vitro* antitumor activity. 2) Determination of *in vivo* antitumor activity 3) Acute toxicity testing for the most potential complex in BALB/c mice.
CHAPTER II

MATERIALS, METHODS AND INSTRUMENTS

Materials Required:

Cell line studies: HeLa and DLA cell lines were obtained from NCCS Pune, India and maintained at Laboratory of Tumor Immunology and Functional Genomics, Regional Cancer Centre, Thiruvananthapuram, India.

DMEM Media preparation:

DMEM

NaHCO3

HEPES

13.5g

3.75g

1.95g

Dissolved in 1000 ml of sterile distilled water. Added 500μL each of anti-mycotic and antibiotic solution. Filter sterilized, sealed and kept at 4oc.

FCS-DMEM Media

5-20% Fetal Calf Serum was added to the sterile DMEM media prior to use.
Phosphate Buffered Saline (PBS)

NaCl 8g
KCl 0.2g
Na2HPO4 1.44g
KH2PO4 0.02g
Distilled Water 1000 ml

Sterilized by autoclaving at 121°C for 15-20 minutes.

PBS-EDTA

EDTA 20g
PBS 100ml

Trypsin Solution

Trypsin 200mg
PBS-EDTA 100ml

Sterile distilled water and the whole solution was sterilized by filtration.

DMSO – Dimethyl Sulfoxide
MTS-PMS solution

Stock PMS (phenazine methosulfate) was dissolved in PBS at a concentration of 0.92 mg/ml DPBS (Dulbecco’s Phosphate Buffered Saline). The solutions were then stored in light-protected tubes at -20°C. MTS and PMS detection reagents were mixed, using a ratio of 20:1 (MTS: PMS), immediately prior to addition to the cell culture at a ratio of 1:5 (detection reagents: cell culture medium).

Ethidium Bromide /Acridine Orange Dual Stain (100X Stock)

Ethidium Bromide 5 mg
Acridine Orange 1.5 mg

Dissolved in 0.1ml 95% ethanol and made up to 5 ml using distilled water. Thaw a 100 uL aliquot of the 100X Stock Solution and dilute 1/100 in phosphate buffered saline. Mix well. Store in an amber bottle at 4°C for up to one month. Add equal 25 µL volumes of cell suspension and ethidium bromide/acridine orange solution to a tube. Mix gently.

100mM TRIS

12.11g in 60ml distilled water. Adjusted pH to 8 and made upto 100ml and autoclaved.
100mM EDTA

3.722g in 100ml distilled water, autoclaved and stored at 4°C.

**Lysis Buffer**

100mM Tris 0.5ml.
100mM EDTA 0.2ml
Triton X 100 0.01ml
Distilled water 0.29ml

**TBE Buffer (10X)**

Tris 108g
Boric Acid 55g
0.5M EDTA 40ml

pH adjusted to 8 and made up to 1L with distilled water.

**Loading dye**

0.25 % Xylene cyanol ; 0.25% Bromo phenol blue. Dissolved in 30% glycerol.

**Agarose** : 2% ; 1.4 g for 70 ml TBE

**Ethidium bromide**: 3.5 µL of 10mg/ml stock for 70 ml Agarose-TBE mixture

**RNase** : 2µL of 10mg/ml for 500µL sample
Proteinase: 3µL of 20mg/ml for 500µL sample

Molecular weight marker (100bp).

Chemicals

The chemicals used for the preparation of complexes were of Analar grade quality - Merck. Hydroxyacetophenone, Amino thiophenol, Aminophenol, Benzil etc were obtained from Sigma, USA. Complexes were synthesized in our laboratory and characterized by elemental analysis, magnetic susceptibility and spectral studies as described in Part I. Solvents were purified by standard methods.

Zn-HAPATP: Zn-2Hydroxyacetophenone 2-Aminothiophenol

Cu-BAP: Cu-Benzil 2-Aminophenol

Cu-HAPAP: Cu-2Hydroxyacetophenone 2-Aminophenol

Cu-HAPATP: Cu-2Hydroxyacetophenone 2-Aminothiophenol

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<th>Ligands</th>
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<td>L₁-2 - Hydroxyacetophenone 2-amino thiophenol</td>
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<tr>
<td>M₂ - Nickel</td>
<td>L₂-2 - Hydroxy acetophenone 2-amino phenol</td>
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<td>M₄ - Zinc</td>
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Antitumour Animal studies and Toxicity testing:

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<td>Amphotericin</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>Pencillin</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>Cyclophosphamide (CYP) - Ledoxan</td>
<td>Dabur India Ltd.</td>
</tr>
</tbody>
</table>

MTS - Non-radioactive cell proliferation assay kit

MTS - 2ml; PMS - 100μl

RBC lysing solution

\[
\begin{align*}
\text{NH}_4\text{Cl} & \quad 8.29\text{g} \\
\text{KHCO}_3 & \quad 1\text{g} \\
\text{Na}_2\text{EDTA} & \quad 37\text{mg} \\
\text{Dist. Water} & \quad 1\text{ L} \\
\text{pH} & \quad 7.2
\end{align*}
\]
**Leishman’s Stain**

Leishman’s stain 150 mg
Methanol 100 ml

Stain was constantly stirred overnight, filtered and used.

**Turk’s fluid**

Glacial acetic acid 1.5 ml
Crystal violet 1 drop
Distilled water 98.5 ml

Above preparation was stirred overnight, filtered and stored in a dark bottle until use.

**Hematoxylin**

Hematoxylin 6.4 g
Ammonium alum (NH₄)₂SO₄.Al₂(SO₄)₃.24(H₂O) 60 g
Ethanol 200 ml
Glycerol 160 ml
Distilled water 640 ml
Mix for quite a while (about 2-4 hrs). Allow the stain to ripen in the dark for 6-8 weeks before using. Although it will work immediately after mixing the resulting stain is somewhat dull.

**Eosin stain**

- Eosin Y 1 g
- 70% Ethanol 1 L
- Glacial acetic acid 5 ml

**Working solution:** Dilute Eosin stock solution 1:1 with 70% ethanol, then add 2-3 drops of glacial acetic acid.

**Acid Alcohol**

- Conc. HCl 4 ml
- 95% Ethanol 396 ml

**Blueing Agent**

- Sodium bicarbonate 1 g
- Distilled water 1 L
Methodology

Establishing Cell Cultures from Frozen Cells

- Placed 10 ml of growth medium in a 15-ml conical tube.

- Thawed the frozen cryovial of cells within 40–60 seconds by gentle agitation in a 37°C water bath.

- Removed the cryovial from the water bath and decontaminated the cryovial by immersing it in 70% (v/v) ethanol (at room temperature).

- Transferred the thawed cell suspension to the conical tube containing 10 ml of growth medium.

- Collected the cells by centrifugation at 200 × g for 5 minutes at room temperature.

- Removed the growth medium by aspiration.

- Resuspended the cells in the conical tube in 5 ml of fresh growth medium.

- Added 10 ml of growth medium to a 75-cm² tissue culture flask and transferred the 5 ml of cell suspension to the same tissue culture flask. Placed the cells in a 37°C incubator at 5% CO₂.

- Monitored cell density daily. Cells were passaged when the culture was at 50% confluency.
Preparation of Cell Liquid Nitrogen Stock

- When growing cells for the production of liquid nitrogen stock, cultures were maintained at 50% confluence.

- Collected cells from a healthy, log-phase culture. Removed the culture medium by aspiration. Trypsinized cells for 1–3 minutes in 1.5-ml of Trypsin-EDTA solution.

- Diluted the cells with 8.5 ml of growth medium. The serum in the medium inactivates the trypsin. Transferred the suspension to a 15-ml conical tube, then collected the cells by centrifugation at 600 × g for 5 minutes at room temperature.

- Removed the medium by aspiration. Resuspended the cell pellet in a minimal volume of growth medium (containing 10% fetal bovine serum). Counted the cells present in an aliquot of the resuspension using a hemocytometer.

- Diluted the cell suspension to 1 × 10^6 cells/ml in freezing medium, then dispensed 1-ml aliquots of the suspension into 2-ml cryovials.

- Frozen the cell aliquots gradually by placing the vials in a shell freezer and then placed in a −80°C freezer overnight.

- Transferred the vials of frozen cells to liquid nitrogen for long-term storage.
Passaging of Cells

- Removed the growth medium by aspiration. Washed cells once with 10 ml of phosphate-buffered saline.

- Trypsinized cells for 1–3 minutes in 1.5-ml of Trypsin-EDTA solution.

- Diluted the cells with 8.5 ml of growth medium to inactivate the trypsin.

- Transferred 1 ml of the cell suspension to a fresh 75-cm² tissue culture flask and added 9 ml fresh growth medium. Placed the cells in a 37°C incubator at 5% CO₂.

- Monitored cell-density daily.
A. Fluorescent microscopic picture of HeLa cells

B. Fluorescent microscopic picture of DLA cells
Preparatory Phase for Cytotoxicity Assay

a. HeLa Cells

The cultured HeLa cells in T-75 flask were trypsinized, detached and centrifuged. The pellet was dissolved in FCS-DMEM media. 10 µl of the suspension was taken in hemocytometer and counted for average number of cells per ml. The required number of cells per well for cytotoxicity assay was 2500-5000 cells/well and calculated accordingly the required volume for 96 microtitre wells.

b. DLA Cells

The DLA cells were aspirated from the peritoneal cavity of Balb/C tumour mice, collected in PBS and cold-centrifuged. The pellet was dissolved in FCS-DMEM media. 10 µl of the suspension was taken in hemocytometer and counted the average number of cells per ml. The required number of cells per well for cytotoxicity assay was 5000 cells and hence calculated the required volume for 96 microtitre wells.

Calculation:

Average number of cells counted = 208 x 10³ cells/ml or 208x10³ cells/100µl

Required 5000 cells per well in 100 µl;

For 5000 cells/ well, the volume required is = (100 x 5000) / (208 x 10³) µl

Therefore for 96 wells = (100 x 5000 x 96) / (208 x 10³) µl
Serial dilution of Transition Metal Complexes - 2000µg/ml to 0.98µg/ml
Assessment of Cytotoxicity

Transition metal complexes (TMC’s) were dissolved in DMSO and serially diluted using micropipettes. Cytotoxicity was then assessed using the Trypan Blue Dye exclusion staining and MTS non-radioactive cell proliferation assay.

Short-Term Cytotoxicity by TMC’s using Trypan Blue

This dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, Eosin, or propidium, whereas dead cells do not. For Trypan Blue staining, HeLa/DLA cell lines were incubated with varying concentrations of the TMC’s starting from 2000 μg/mL to 0.98μg/mL for 3 hours and 1% Trypan Blue was added for a minute and counted. Viable cells exclude dye while non-viable cells are blue colored. Cytotoxicity is determined by calculating percentage cell deaths. In the case of HeLa cells, the cells are detached by trypsinization prior to evaluating.

Determination of Cytotoxicity by TMC’s in HeLa cells using MTS assay:

MTS assay uses the soluble tetrazolium salt, MTS, and it is versatile and offers several advantages over MTT and other cytotoxicity assays due to
the solubility of the MTS formazan product in culture medium. MTS is chemically reduced by cells into formazan, which is soluble in culture medium. The measurement of the absorbance of the formazan can be carried out using 96 well microplates at 490nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells; the intensity of the produced color is a good indication of the viability of the cells.

HeLa cells (5000 cells / well), control 1 (medium only) and control 2 (medium + cells) were seeded in triplicates in 96 well microtitre plates and incubated for attachment at 37°C in 5%CO₂ incubator for 12 hours. After 12 hours, 100 µl of different concentrations of TMC’s (concentration ranging from 2000 µg/ml to 0.98 µg/ml) were added to each well excluding the control wells. The plates were then incubated at 37°C in 5% CO₂ incubator for 48 hours. 20 µL of MTS-PMS solution was added and incubated in dark for another 4 hours and absorbances were recorded at 490 nm using ELISA plate reader. Graph was plotted with percentage cell cytotoxicity on Y-axis and concentration of TMC’s (µg/ml) on X-axis.
Percentage cell viability is calculated using the formula (James Kumi-Diaka et al., 2004).

\[
\% \text{ Cell viability} = \frac{A_T - A_B}{A_C - A_B} \times 100
\]

Where \( A_T \) is the absorbance of test; \( A_C \) is the absorbance of control; \( A_B \) is the absorbance of the blank.

\[
\% \text{ Cell cytotoxicity} = 100 - \% \text{ cell viability}
\]

**IC\(_{50}\) determination**

IC\(_{50}\) value (inhibitory concentration at 50%; the concentration of the test substance required to reduce the light absorbance capacity of exposed cell cultures by 50%) was calculated using the formula: AbsIC\(_{50}\) = (AbsIC\(_{100}\) + AbsIC\(_{0}\)) / 2. The mean absorbance generated by the "medium-only" control is denoted as IC\(_{0}\). The mean absorbance generated by the "medium with cell" control is denoted as IC\(_{100}\).

**Determination of Cytotoxicity by TMC’s in DLA cells**

DLA cells (5000 cells / well), control 1 (medium only) and control 2 (medium + cells) were seeded in triplicates in 96 well microtitre plates and incubated at 37°C in 5%CO\(_2\) for 6 hours. After 6 hours, 100 µl of different concentrations of TMC’s (concentration ranging from 2000µg/ml to
0.98µg/ml) were added to each well excluding the control wells. The plates were then incubated at 37°C in 5% CO₂ incubator for 48 hours. 20 µL of MTS-PMS solution was added and incubated in dark for another 4 hours and absorbances were recorded at 490 nm using ELISA plate reader. Graph was plotted with percentage cell cytotoxicity on Y-axis and concentration of TMC’s (µg/ml) on X-axis. Percentage cytotoxicity and IC₅₀ was determined as mentioned before.

**Deducing cell morphology by Ethidium Bromide/Acridine Orange Dual Stain**

Acridine orange/Ethidium bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis. Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. Consequently, ethidium bromide is
often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

Drug induced apoptosis and necrosis were determined morphologically after labelling with acridine orange and ethidium bromide (Duke & Cohen, 1992). DLA cells ($10^6$ cells in 100µl) were cultured with 100 µl of different concentrations of Transition Metal Complexes at 37°C in 5% CO$_2$ incubator for 24 hours. Cells were pelleted and processed for apoptosis assay. Thawed 100 uL aliquot of the 100X A.O/Et.Br stock and diluted 1/100 in phosphate buffered saline. Added equal 25 µL volumes of treated/non-treated DLA cell suspension and ethidium bromide/acridine orange solution to a tube and observed under fluorescent microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide.
Determination of DNA Fragmentation using DNA Ladder Assay

Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 basepairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis known as DNA laddering. In DNA laddering assay, small fragments of oligonucleosomal DNA is extracted selectively from the cells whereas the higher molecular weight DNA stays associated with the nuclei. The isolated DNA is separated by electrophoresis and visualized using ethidium bromide.

Isolation of DNA

DLA cells (10⁶ cells in 100µl) were cultured with 100 µl of different concentrations of Transition Metal Complexes at 37°C in 5% CO₂ incubator for 48 hours. Cells were pelleted and processed as follows.

- Pellet was dissolved in 1 ml lysis buffer.
- Added 0.02 ml of 10% SDS.
- Then added 2µl RNase (10mg/ml).
- Incubated at 56°C for 2 hours in dry bath.
- Added 3 µl proteinase K (20mg/ml).
- Incubated at 37°C for 2 hours (or overnight) in dry bath.
• Equal volume of isopropanol was added and centrifuged @ 10,000rpm at 4°C.

• Dried and dissolved in TE buffer (10-50μl).

• Stored at -20°C for further use.

DNA Laddering Assay

• 1.4g of Agarose was weighed and boiled to dissolve it in 70ml 1X TBE buffer (Tris-Borate-EDTA) to prepare 2% gel.

• Allowed to lower the temperature to about 50°C and 3.5 μl of 10mg/ml stock ethidium bromide was added and mixed well.

• Agarose-TBE was then poured onto a clean gel plate with comb inserted.

• After 5-10 minutes, when the gel was set, more 1x TBE buffer (~300ml) was poured and the comb was removed to load the mixture [5μl sample + 2μl of 6X loading dye (Bromophenol blue)].

• Power pack was connected and the voltage was set to 100V.

• After the initial movement of the sample + dye, a steady voltage of 80V was maintained for 2-3 hours.

• Analysed for DNA fragmentation in Gel Documentation system and ladder obtained is recorded.
Determination of Anti tumour activity in experimentally tumour induced mice

Experimental animals

Studies were carried out using inbred strains of 6-8 weeks old BALB/c mice weighing 25±4 g housed in polypropylene cages with dark/light cycle (14/10 h). The animals were housed under controlled temperature and hygienic conditions. All animals were fed with standard pellet diet and water ad libitum. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Development of Lymphoma

Dalton’s lymphoma cell lines were maintained in the inbred BALB/c mice. The lymphoma cells were collected from the peritoneum in cold PBS in asceptic conditions were pelleted after centrifugation. The cells were counted in a hemocytometer. Each experimental mouse received 1x10⁶ cells, intraperitoneally. The lymphoma was allowed to grow invivo for about 14 days. At the end of the experimental period the animals were sacrificed.
Normal mice (above) & DLA mice (below) after 17 days of tumour induction
Experimental Design

Transition metal complexes (TMC’s) were dissolved immediately before intraperitoneal administration in 0.9 % saline. Inbred BALB/c mice weighing 25 ± 4g were divided into 6 groups, each consisting of 10 animals. Group I- Control (PBS), Group II- TMC (e.g Zn-HAPATP at a standardized dose of 5 mg/kg b.wt i.p. for 14 days), Group III served as DLA control (10⁶ cells injected /animal), Group IV- DLA + TMC, Group V- DLA+CIS (2mg/kg b.wt. per animal i.p. post tumour inoculation) and Group VI-DLA+CIS+TMC. 24 hours after the last dose i.e.; after 14th day, animals were sacrificed by cervical dislocation and the parameters were assessed.

Determination of Effect of TMC on Hemoglobin Concentration

Principle: Ferricyanide forms methemoglobin with hemoglobin, which is converted to cyanomethemoglobin by cyanide, which has an absorption at 540 nm.

Procedure: Blood (20μl) obtained from tail vein was mixed with 5ml of Drabkins reagent allowed to stand for 5 min at room temperature. Optical density (OD) was measured against regent blank. Haemoglobin content was calculated using the formula,

\[ g \% \text{ of Hb} = \frac{O.D. \text{ of the Test} \times 251 \times \text{Conc. of Std.}}{O.D. \text{ of the Std.} \times 1000} \]
Determination of Effect of TMC on Total Leukocyte Count

**Principle:** Blood was diluted in Turk’s fluid, which contains a weak acid (acetic acid) to lyse RBC and a stain (crystal violet) for staining leucocytes. The number of cells in the large four corner squares of hemocytometer was counted.

**Procedure:** Blood was collected from tail vein and diluted with 3% acetic acid in saline solution so as to lyse all the erythrocytes. Dilutions of 1:100 or 1:20 were prepared; the Unopette ® micro collection system ([Becton Dickinson and Co.](https://www.bd.com)) was used to prepare the dilutions. All samples were mixed using a Vortex prior to evaluating. Leucocytes were loaded on to the Neubauer hemocytometer and four large squares counted under a microscope using 10x objective. The Total White Blood Cells count was determined using the formula.

\[
\text{Total count} = \frac{\text{Number of cells counted (N) \times \text{Dilution factor} \times \text{Depth factor}}}{\text{Area counted}}
\]

**Determination of Effect of TMC on Differential Count (D.C.) of leucocytes**

Differential staining helps to study the morphology of leucocytes. There are five types of white blood cells.
- Neutrophils 40-75 %
- Eosinophils 5 %
- Basophils 0.5%
- Lymphocytes 20-50%
- Monocytes 1-5%

Normal mice differential staining show lymphocytes as the major population comprising 65-80% followed by neutrophils 10-20%, monocytes 2-6%, eosinophils 1-2% and basophils 0-2%.

**Procedure**

- A thin film of blood was made by smearing the drop of blood evenly across the glass slide using a glass spreader.
- Air dried and flooded the smear with 3 drops of Leishman’s stain.
- After 3 minutes, flooded the smear with distilled water.
- Kept for 10 minutes, washed with tap water
- Observed under oil immersion 100 x objective.
The cells observed were;

**Neutrophils:** These granulocytes have segmented nuclei, with 2-5 lobes connected together by thin strands of chromatin and has a C shaped nucleus.

**Eosinophils:** They are the granulocytes having bilobed nuclei.

**Basophils:** Characterized by large cytoplasmic granules with obscure nuclei.

**Monocytes:** Agranulocytes having U shaped with reticular appearing chromatin.

**Lymphocytes:** Agranulocytes having a deeply stained nucleus which may be eccentric in location having small amount of cytoplasm.

**Determination of Effect of TMC on Bone Marrow Cellularity**

Bone marrow cells were obtained by flushing the femur bone with DMEM media containing 10% FBS and made a single cell suspension. Bone marrow cells were stained and checked for viability using Trypan blue in a hemocytometer and expressed as total live cells/ femur.
Determination of Effect of TMC on Lymphocyte Proliferation by MTS Assay

**Principle:** In 1968, Boyum described methods for isolation of mononuclear cells from circulating blood and bone marrow. In general, these procedures employed mixtures of polysaccharide and a radio opaque contrast medium. The solution contains sodium diatrizoate, adjusted to a density of $1.077 \pm 0.001$. This medium facilitates rapid recovery of viable lymphocytes from small volumes of blood, on centrifugation. It is usually employed as the initial isolation step prior to enumeration of T, B and null lymphocytes.

Lymphocyte proliferation assay (LPA) measures the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. CD4+ lymphocytes proliferate in response to antigenic peptides in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). This proliferative response of lymphocytes to antigen *in vitro* occurs only if the patient has been immunized to that antigen, either by having recovered from an infection with the microorganism containing that antigen, or by having been vaccinated. Therefore, some normal individuals may not respond to a given antigen, but most people will respond to at least one of several common
microbial antigens. Antigen-specific T-cell proliferation is a major technique for assessing the functional capacity of CD4+ lymphocytes to respond to various stimuli. Almost everyone’s lymphocytes can be stimulated to proliferate nonspecifically by stimulating them \textit{in vitro} with the mitogen phytohemagglutinin (PHA) or pokeweed mitogen (PWM), or the antibody anti-CD3 (Phillip Wong and Pamer EG, 2001). However, these substances provide strong stimuli that are not antigen specific, and usually do not discriminate as well as antigens in reflecting different levels of immunodeficiency.

\textbf{Isolation of Lymphocytes and Culturing}

Splenic lymphocytes were obtained by gentle disruption of the spleen by passing through a wire mesh with DMEM medium with 10% FCS at 37°C under 5% CO2 in air. In the case of non-availability of spleen, peripheral blood cells diluted with DMEM was layered on the Ficoll Hypaque plus solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. Because of the lower density of erythrocytes and granulocytes, the lymphocytes are found at the interface between the plasma and the Ficoll Hypaque plus with other slowly sedimenting particle (Platelets and monocytes). Lymphocytes were then recovered from the interface and
subjected to a short washing step with a balanced salt solution to remove any platelets, Ficoll Hypaque plus and plasma.

**Principle:** MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial succinic dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a purple formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the purple formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multi well scanning spectrophotometer (ELISA reader). The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Reagent (Promega, USA) is prepared by combining two solutions, MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (novel compound different from MTT without the need of adding DMSO to solubilise) and an electron coupling reagent, phenazine methosulfate (PMS). The mixed reagent is then added to cells. During the assay, MTS is
converted to a soluble formazan product. Samples are read after a 1–4 hour incubation at 490 nm.

**Procedure:** Splenocytes were prepared by disrupting the spleen with DMEM medium and layered with the diluted blood over ficoll (1 ficoll: 3 blood). After 20 minutes of centrifugation at 2000 rpm to separate cells from the debris, upper layer is drawn off using a clean pasteur pipette, leaving the lymphocytes layer undisturbed at the interface. Using a clean pasteur pipette the lymphocyte layer was transferred to a clean centrifuge tube and washed it with DMEM media and cells were counted using a haemocytometer (Cheesbrough and Mac, 1976).

The isolated lymphocytes were suspended in DMEM medium to adjust the cell count (1x10^6 cells/ml). After adding sufficient amount of cell suspension (≈200 μl) per well in 96 well plates, 10 μl of PHA is added in triplicates (without mitogen serves as control) and the microtitre plates were incubated for 48-72 hours at 37°C. 20 μl of the combined MTS/PMS solution is pipetted into each of the 96 well assay plate containing 210 μl volume of lymphocites and PHA. The amount of purple formazan was determined by measuring the absorbance at 490 nm after 4 hours of incubation in dark and cell proliferation was calculated by the formula T/C x 100, where T-Test
O.D., C-Control O.D. The result obtained is the proliferation index or expressed as percentage relative cell viability.

**Tumour Volume, Median Survival Time (MST) and Percentage Life Span Analysis**

Dalton’s Lymphoma Ascites were injected in mice to develop tumour and the tumour volume were analysed on 7th, 14th and 21st days after *i.p.* treatment of TMC at a dose of 5mg/kg body weight once daily starting from day 1 till the day of sacrifice. The volume in tumour induced as well as TMC administered animals were measured using a syringe and puncturing the peritoneal cavity so that no tumour cells are spilled out. The volume measured in ml. is noted on the respective days. The MST and % ILS was calculated by the formula (Angela Garofalo et al., 2003).

\[
\text{Median Survival Time; } MST = (\text{Date of 1st death} + \text{Date of last death}) / 2
\]

\[
\text{Percentage increment of life span; } %\text{ILS} = (\text{MST of test} / \text{MST of control}) - 1 \times 100
\]

**Acute Toxicity Testing of TMC**

Mice were acclimatized to laboratory conditions for 7 days prior to initiation of dosing. They were randomly assigned to cages and the individual animal was fur marked with picric acid. The females were
nulliparous and non-pregnant. Mice were assigned to treatment groups of 3 males and 3 females. The mice were deprived of feed for 16 hours before and 3 hours after administration of the test substance. The test substance, TMC at doses of 10mg/kg, 20mg/kg and 40mg/kg body weight were administered i.p. to mice of both sexes once at the starting day of experiment.

Observations of pharmacotoxic signs were made at 10, 30, 60, and 120 minutes and at 4 and 6 hours after dosing during the first day and daily thereafter for 7 days. The time of onset, intensity, and duration of these symptoms, if any, was recorded. All animals were observed twice daily for mortality during the 7-day period of study and LD-50 was also recorded. The weight of each mouse was recorded on days 0, 3 and 7. The group mean body weights were calculated. Animals’ dead were immediately dissected to extract the serum for liver and renal function tests. Tissues and organs were preserved in 10% neutral buffered formalin for histopathological analyses. Histopathological and alteration in serum enzymes were done at DDRC, Thiruvananthapuram, India.

**Histopathology of liver and kidney in mice**

Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei
blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red. Histopathological observations of liver and kidney were carried out in control and animals treated at the highest dose level of 40mg/kg.

**Protocol for H & E Staining:**

- Paraffin embedded slides are deparaffinized and rehydrated, frozen or vibratome sections are best mounted on slides and rehydrated;
- Slightly overstain the sections with hematoxylin, usually 3-5 minutes, depending upon section thickness and fixative (up to 20 min. if solution is not fully ripened);
- Remove excess stain in tap water, 2 min.
- Differentiate and destain a few seconds in acidic alcohol until sections look red, usually 4-5 dips.
- Rinse briefly in tap water to remove the acid;
- Blue in bicarbonate until nuclei stand out sharply blue, about 2 min.;
- Rinse in running tap water, 8 min.; Dehydrate and clear, or stain with eosin.
Eosin staining:

- Take hematoxylin stained slides from the last tap water rinse and place in 70% ethanol for 3 min.;
- Place slides in eosin for 2 min.;
- Take slides through 3 changes of 95% ethanol, 5 min. each;
- Then transfer to the first absolute ethanol of the clearing series.

**Testing hematoxylin stain solution to see if it is still usable:** Add several drops of stain to tap water (not distilled or deionized). If they turn bluish-purple immediately, it is satisfactory. However, if they change slowly, stays reddish or brownish, then the stain should be discarded.

**Statistical Analysis**

Statistical analysis was done using Analysis of Variance (ANOVA) followed by Tukey Kramer multiple comparisons test.
CHAPTER III

ANTI TUMOUR STUDIES ON SOME TRANSITION COMPLEXES OF SCHIFF BASES

The ability of medicinal formulations containing metal ions and related materials to cure a variety of diseases were well known from ancient times. In this contest it is logical to consider the extension of the use of coordination chemistry for medicinal purpose incorporating metal ions. Literature survey revealed that Schiff base metal complexes were well known for their antitumour activity. In continuation of the work carried out in our laboratory we tried to screen and study the antitumour activity of some selected metal complexes.

16 metal complexes of Co(II), Ni(II), Cu(II) and Zn(II) and their ligands namely 2-Hydroxyacetophenone 2-Aminothiophenol (HAPATP), 2-Hydroxyacetophenone2-Aminophenol (HAPAP), Benzil 2-Aminothiophenol (BATP) and Benzil 2-Aminophenol (BAP) were tested for their short and long term in vitro cytotoxic action using DLA and HeLa tumour cell lines. Based on the studies four complexes were selected for the toxicity and apoptosis studies. Two metal complexes were found to be more active. Among the two metal chelate, Zn-HAPATP (M₄L¹) was selected to study the effect on solid tumour reduction in BALB/c mice.
RESULTS

1. Effect of Transition Metal Complexes on Short Term *Invitro* Cytotoxicity

Results of short term cytotoxicity as obtained by Trypan Blue staining indicated 4 out of 16 complexes in about 12 concentrations screened showed promising cytotoxic activity against DLA (Table III.3.1) as well as HeLa (Table III.3.2) cell lines in about 3 hours. The cytotoxicity was more severe in DLA cells which brought about 90% of the cell death by M\(^4\)L\(^1\) complex followed by M\(^4\)L\(^4\) (75%), M\(^3\)L\(^2\) (60%) and M\(^3\)L\(^1\) (55%). In the case of HeLa cells, the cytotoxicity was observed in the order M\(^4\)L\(^1\) (60%) > M\(^3\)L\(^1\) (57%) > M\(^3\)L\(^2\) (56%) > M\(^3\)L\(^4\) (52%).

2. Effect of Transition Metal Complexes on Long Term *Invitro* Cytotoxicity

Results of long term cytotoxicity of Transition Metal Complexes by MTS assay confirmed cytotoxicity of these 4 complexes in the order Zn-HAPATP (M\(^4\)L\(^1\)) > Cu-BAP (M\(^3\)L\(^4\)) > Cu-HAPAP (M\(^3\)L\(^2\)) > Cu-HAPATP (M\(^3\)L\(^1\)) in both the cell lines (Figure. III.3.1). M\(^4\)L\(^1\) showed the maximum cytotoxic ability in DLA as well as HeLa cell lines in all the doses tested. However, these 4 complexes were non-toxic in normal lymphocytes. The IC\(_{50}\) values obtained against DLA cells for M\(^4\)L\(^1\) (62.5\(\mu\)g/mL) was the lowest and M\(^3\)L\(^1\) (1350 \(\mu\)g/mL) was the highest (Table III.3.3) whereas IC\(_{50}\) values against HeLa for M\(^4\)L\(^1\) (225 \(\mu\)g/mL) was the lowest and M\(^3\)L\(^1\) (1450 \(\mu\)g/mL) was the highest (Table III.3.3).
Figure III.3.1: Determination of Cytotoxicity by TMC’s in DLA/HeLa cells

DLA/HeLa cells (5000/well) were cultured in the presence of TMC’s at 37°C in 5% CO2 incubator for 48 hours. 20 µL of MTS-PMS added, incubated in dark for another 4 hours and absorbances were read at 490nm. Graph was plotted with percentage cell cytotoxicity on Y-axis and concentration of TMC’s (µg/ml) on X-axis. Percentage cytotoxicity was calculated according to James-Kumi Diaka et al, 2004. IC50 was calculated from the obtained graph.
3. Effect of M\textsuperscript{4}L\textsuperscript{1} and M\textsuperscript{3}L\textsuperscript{4} on Morphological Assessment of Apoptosis

DLA cells untreated after 12, 18 and 24 hours showed the green fluorescence representing 100% viability (Figure III.3.2A). DLA cells treated with 62.5μg/mL of M\textsuperscript{4}L\textsuperscript{1} showed varying degrees of evident apoptosis in a time dependent manner. Nuclear and cytoplasmic condensation with blebbing of the plasma membrane, and formation of apoptotic bodies (Orange-red fluorescence) were prominent at the end of 18\textsuperscript{th} and 24\textsuperscript{th} hour; the latter being severe (Figure III.3.2C and III.3.2D). However, some cells showed necrosis (red fluorescence) as the membrane integrity was lost due to cell rupture releasing noxious cellular contents (Figure III.3.2C). M\textsuperscript{3}L\textsuperscript{4} at an effective concentration of 125μg/mL showed similar but less intense effects at the end of 12\textsuperscript{th}, 18\textsuperscript{th} and 24\textsuperscript{th} hours (Figure III.3.3B, III.3.3C and III.3.3D).

4. Effect of Transition Metal Complexes on Tumor cell DNA fragmentation

Electrophoresis of DNA samples showed that M\textsuperscript{4}L\textsuperscript{1} and M\textsuperscript{3}L\textsuperscript{4} treated cells exhibited extensive double stranded DNA breaks, as evident by a ladder appearance, while the DNA of other TMC’s treated DLA cells exhibited no laddering (Figure III.3.4). The positive control used for the study was a 100bp molecular weight marker. DLA cells treated with 62.5μg/mL M\textsuperscript{4}L\textsuperscript{1}, and 125μg/ml M\textsuperscript{3}L\textsuperscript{4} for 48 hours showed significant DNA fragmentation as evident from characteristic DNA laddering (Figure III.3.4).
5. Effect of M^4L^1 on Blood picture and Bone marrow cellularity in mice

Intraperitoneal administration of M^4L^1 for a period of 14 days instigated a significant (P<0.001) increase of hemoglobin in DLA induced mice, whereas normal and Cisplatin treated DLA mice did not benefit on M^4L^1 treatment (Table III.3.4). Total count and bone marrow cellularity were significantly improved (P< 0.001) on M^4L^1 treatment in tumour as well as Cisplatin treated tumour mice (Table III.3.4). But, these values were non-significant even after M^4L^1 treatment in normal mice. The differential staining revealed that majority of the cells are lymphocytes unlike neutrophils in the human blood. M^4L^1 administration showed a responsive increase in the counts of lymphocytes and neutrophils in the DLA mice (P<0.001) and not in other groups (Table III.3.4).

6. Effect of M^4L^1 on mice lymphocytic response to PHA

In control mice administered with M^4L^1; culturing splenocytes with PHA showed a proliferation index of 1.41 (P<0.01) while DLA mice showed greater significance (P<0.001) on M^4L^1 treatment in the rates of lymphocyte proliferation. However, Cisplatin also showed a proliferation index of 1.38 which unfortunately showed lesser effects on M^4L^1 treatment (Figure III.3.5).
7. Effect of M⁴L¹ on Tumor volume, MST and percentage increment in life span

DLA induced mice on M⁴L¹ treatment showed less promising regression on 7th and 14th day, but until 21st day (P<0.001) where it significantly reduced tumor volume (Figure III.3.6). However, the situation is just reverse in the Cisplatin treated tumor mice, where it decreased the tumor burden on all days, when compared to other groups. The standard anticancer drug itself is being effective against established tumours. But, the combination treatment of M⁴L¹ and Cisplatin could effectively bring down the tumour volume slightly, although not upto that of Cisplatin alone. The MST in days (21) with respect to tumour control (17.8) was significant (P<0.05) which also could enhance the lifespan upto 18 % with M⁴L¹ treatment alone (Table III.3.5). Here also, the anticancer drug – Cisplatin could effectively increase the lifespan upto 34.83 %, but only slightly more than the M⁴L¹ and Cisplatin combination treatment (33.14%).
8. Acute toxicity testing of M₄L¹ in mice

Intraperitoneal administration of M₄L¹ at 20 mg/kg b.wt. in mice did not show any toxic effects and all of them were alive even after 7 days while at 40mg/kg body weight, 50% of the mice died within first 3-12 hours; being the LD50 of the drug M₄L¹ and rest 50% of animals recovered the following day. However at 20 mg/kg, mild hypoactivity was observed for 4 hours post-administration and all animals appeared normal by the following day and throughout the 7-day observation period. Upto 20 mg/kg dose injected, the complex M₄L¹ appeared relatively safe. The serum enzyme levels showed a 3 fold increase in the 40 mg/kg b.wt. group which reciprocated toxic effects to liver and kidney, thus causing the death of some animals. At the same time, AST/SGOT (Aspartate amino transferase/ Serum Glutamate Oxaloacetate Transaminase), ALT/SGPT (Alanine amino transferase/ Serum Glutamate Pyruvate Transaminase) and ALKP (Alkaline phosphatase) were within safer limits upto doses of 20mg/kg b.wt (Figure III.3.7). Histopathology of liver showed intact portal triad with normal hepatocytes (Figure III.3.8A) which on intraperitoneal administration of 40mg/kg M₄L¹ ruptured liver chords with congestion of hepatocytes and hyperchromatic pyknotic nuclei (Figure III.3.8C). Kidney also showed normal equally spaced glomerulus (Figure III.3.9A) which underwent tubular degeneration and bleeding in the Bowman’s capsule (Figure III.3.9C) at a dose of 40mg/kg.
Figure III.3.7: Effect of M4L1 on Serum Enzymes

M4L1 was administered intraperitoneally at doses of 20mg/kg and 40mg/kg/animal (n=6) once and observed for 7 days. In dead mice, blood was immediately drawn and liver function tests were performed. Data were expressed as mean ±SEM. Statistically significant differences at ***P<0.001, *P<0.05, ns–non-significant, as compared with control group.
DISCUSSION

Metal ions influence biological phenomena by interacting with organic functional groups on biomolecules, forming metal complexes. A ligand—a compound in which two or more atoms of the same molecule can coordinate with a metal to form a metal chelate. Usually, the metal in the metal chelate is tetra or hexacoordinated, but may be octacoordinated or more highly coordinated depending on the metal ions. The metal chelate will be uncharged, thus the number of acidic groups provided by its ligands will equal the oxidation state of the metal ion (Petering DG, 1980). Usually, the metal ligands will be relatively hydrophobic so as to impart solubility of the metal chelate in non-polar solvents.

Out of the 16 complexes screened, 4 of the transition metal complexes found to be cytotoxic against HeLa as well as DLA cell lines as obtained by Trypan Blue staining (Table III.3.1 and III.3.2). It follows the order Zn-HAPATP (M₄L₁) > Cu-BAP (M₃L₄), Cu-HAPAP (M₃L₂) > Cu-HAPATP (M₄L¹). So, M₄L₁ (Zn-hydroxy acetophenone aminothiophenol) was confirmed to be most potent of the complexes screened with a least IC₅₀ value of 62.5 µg/mL for DLA cells and 225 µg/mL for HeLa cells (Table III.3.3). Zinc and Copper complexes examined in our experiments differ from each other by the Ligands. Each component ligand as well as metal ion
influences the physico-chemical and biological properties of the complexes in different way, which could explain the differences in their cytotoxic effects.

Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. Necrosis is a pathological process which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis is a physiological and controlled process by which unwanted or useless cells are eliminated during development and other normal biological processes (Wyllie AH, 1980). Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed and densely stained chromatin, or membrane-bound apoptotic bodies containing one or more nuclear fragments (Shanker A, 2000). The cell death due to apoptosis was further confirmed as assessed by Acridine Orange/Ethidium bromide dual staining and DNA laddering. Since M^4L^1 was the most potent which was effective against DLA than HeLa as evidenced by cytotoxicity assays, all the essential experiments were done using M^4L^1 on DLA itself. Apoptosis in DLA cells were obtained on M^4L^1 and M^3L^4 treatment at 12, 18 and 24 hours. DNA fragmentation as evidenced by ‘ladder’ was obtained for M^4L^1 and M^3L^4. The low molecular weight and lipid solubility of the Zinc and Copper complexes facilitate penetration of
cell membranes. Depending upon the specific type of complexes used, treatment might have resulted in enhanced immune response to tumours, decreased tumour growth and increased survival of the mice as evidence by its extended life span.

Intraperitoneal administration of M^4L^1 at a dose of 5 mg/kg body weight/animal/day for a period of 14 days was found to be optimal as calculated from the LD_{50} (40mg/kg - dose at which 50% of the animals died) and hence was followed for this evaluation. A dose of 20 mg/kg body weight was seen to have no adverse effects on the animal as per the biochemical examination of various parameters and histological examination of the liver and kidney. Initially, M^4L^1 caused a magenta discoloration of the tail, feet, eyes, nose and internal pinna of the ear almost immediately following injection which was very well stabilized the following day. The intensity of the color appeared to be dose-related. Transient discoloration of highly vascularized external appendages and mild hypoactivity were observed during the first 24 hours post-injection. The discoloration was transient with no apparent signs by 24 hours post-injection. All animals exhibited weight gains during the observation period. Gross examination of internal organs at necropsy was unremarkable. Histopathology of liver showed congestion of hepatocytes and hyperchromatic pyknotic nuclei (Figure III.3.8). Kidney also
showed tubular degeneration and bleeding in the Bowman’s capsule at a dose of 40mg/kg (Figure III.3.9). This could be the reason for the death of the animal. In both the tissues, normal histopathology was observed until 20mg/kg dose. The serum enzymes were elevated only at doses of 40mg/kg indicating that the higher dose was unbearable for the mice which might have resulted in the death of the animal (Figure III.3.7). Mice inoculated with the DLA tumour was evaluated on 7th, 14th, & 21st days of M^4L^1 administration for the effect on body and organ weights, tumour volume, median survival time and percentage increment in life span. Body weights (+1.5 g) and organ weights were marginally increased (except liver and kidney) in the M4L1 treated animals while Cisplatin treated mice showed reduced body weights (~ 2.0 g) with no changes in the organ weights. Reduction in tumour volume was observed in M^4L^1 treated DLA bearing mice at the end of 21st day and initially at the end of 7th day when co-administered with cisplatin (Figure III.3.6). The median survival time and percentage increment in life span also showed slight significant increase on M^4L^1 treatment in the DLA mice (Table III.3.5). 33.14 % increment in the life span was obtained in the combination treatment of Cisplatin with M^4L^1 when compared to DLA control mice, suggesting its promising role in tumour cell inhibition. By NCI criteria, a T/C exceeding 125% and an ILS
exceeding 25% indicate that the drug has significant anti tumour activity (Plowman J, 1995).

In the present study, M\textsuperscript{4}L\textsuperscript{1} (5mg/kg) significantly increased the leukocyte count as well as the bone marrow cellularity in the tumour as well as Cisplatin treated tumour bearing mice, though not effective in the normal mice (Table III.3.4). Bone marrow is the site of hematopoiesis- the prime organ involved in the blood cell production. Many of the hematopoietic growth factors are also produced by the stromal cells, which reside in the close proximity to the precursors which enables to exhibit its functional redundancy and perform biological functions (Paul W, 1999). High amount of hematopoietic cells, particularly lymphocytes are localized in the bone marrow which are able to kill tumor cells or virus infected cells and play a vital role in immune response (Pelczar MJ, 1990). Thus, more amount of lymphocytes which upon presentation of antigenic determinants by the macrophages will initiate a cascade of immune responses involving cytokines, lymphokines etc. imparting anti-tumour potential in the DLA mice. The RBC’s that supply oxygen to the body and WBC’s which fight against infections also originate in the bone marrow. Increase in the WBC and BMC count signifies the ability of the M\textsuperscript{4}L\textsuperscript{1} to mobilize the immune response against the various diseases including infections and cancer. The
statistically significant elevation in the hemoglobin count of M₄L¹ administered DLA bearing mice points out that M₄L¹ alone can contribute to overall immunity in tumour inoculated mice by producing more hematopoetic cells with profound Hemoglobin to circulation. The lymphocyte proliferation was considerably increased in normal as well as tumour bearing mice at a dose of 5mg/kg indicating possible immunostimulant effect (Figure III.3.5). Experimental evidence indicates that M₄L¹ achieved a sizeable peripheral pool of PHA-sensitive, naive T lymphocytes which ensures an improved immune response (Pérez CS, 2001). This shows that the M₄L¹ is able to induce lymphocyte clonal proliferation in normal as well as tumor bearing mice thus making them more reactive to neoplastic challenges. Taken together, the results indicate the anti-tumour immunity imparted by M₄L¹ in the tumour inhibition.

The majority of anticancer drugs act as cytotoxic drugs. While anticancer drugs have proven useful in the treatment of cancer, they are not without harmful effects because of their potential to kill both cancer and normal cells. The serious deleterious effects prompt their discontinuous therapeutic applications (Devasagayam TP, 2002). The major drawbacks of the platinum based anticancer drug, cisplatin, are the serious side effects and acquired drug-resistance, which inevitably increases the drug dosage to
patients. Accordingly, a new anticancer drug that circumvents these clinical inconveniences is strongly desired to improve patient’s quality of life. It is generally believed that cisplatin’s anticancer action is triggered by formation of 1,2-intrastrand crosslinks on DNA, resulting in a severe DNA distortion. Most of the structurally related cisplatin-analogues show cross-resistance to cisplatin, probably due to their similar biological consequences. In this study, cisplatin - 2 mg/kg b.wt. suppressed leucocytes and bone marrow cells in tumour bearing mice (Table III.3.4). When M^4L¹ was administered in combination with cisplatin in DLA bearing mice, WBC counts and BMC were restored to near or above normal levels suggesting the ability of M^4L¹ to counteract myelosuppression. But the combination treatment did not initiate a boosted response to PHA by lymphocytes as well as in bringing down the tumour burden. The clear cut synergistic effect was noticeable only in improving the myelosuppression caused by the Cisplatin. Thus co-administration of these type of protective agents can reduce the undesired toxicity of the antineoplastic agent on healthy cells; thus, it enhances the treatment response. Furthermore, the metal complexes at the concentration checked did not inhibit the proliferation of normal splenocyte and bone marrow cells (data not shown), indicating that the cytostatic effect of the metal complexes was restricted to the tumor phenotype alone. Selective
killing of tumor cells could be attributed to the fact that tumor cells are unable to counter the load of mutations owing to their defective DNA repair mechanism (Rosenberg B, 1985).

Chelation in addition to its selectivity may increase the anti-tumour activity (Furst A, 1963); but this investigation is a primary one and further tests are required to explore its actual mechanism of action and its probable effects on higher animal model and more cancer cell lines. Developing ligands which coordinate to a specific metal ion can build stable complexes with high selectivity. To achieve metal ion selectivity, the design of a ligand with preorganization and complementarity is needed which satisfies the requirements of the metal ion, allowing the formation of the ideal coordination geometry which might have enhanced biological activity (Sigel H, 1998). Apart from this ligands with nitrogen, oxygen and sulphur donor systems inhibit enzyme activity since the enzyme which require these groups for their activity appear to be especially more susceptible to deactivation by the metal ions through coordination.

Therefore, the areas of deficiency, toxicity, and optimum physiological response can be dramatically varied by considering a combination of these variables, as well as design features of the potential ligand which may be altered to tune the delivery of that metal ion into the
biological system. This refinement of the biological properties of metal complexes by ligand modification, along with the design of ligands to alter the homeostasis of endogenous metal ions, will provide many new therapeutic and diagnostic agents over the coming years and will direct medicinal inorganic chemistry into a discipline of central importance in medicine and science.
References


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