INTRODUCTION

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1. INTRODUCTION

1.1 CANCER

The cell forms the basic structural, functional and biological unit of life and is often called the “building blocks of life”. A cell arises from pre-existing cell through the process of cell division where the genetic material is copied and passed along to its offspring. Cell division is responsible for carrying out the three essential functions-reproduction, growth and development and repairing damaged cells. In eukaryotic cells multiplication of cells or cell-division cycle follows a definite sequential steps and it may be divided into two broad stages- Interphase, comprising of G₁ phase (Gap 1) during which cell increases in size, the S phase (synthesis) during which DNA replication occurs, G₂ phase (Gap 2) during which proteins are synthesised in preparation for mitosis while cell growth continues, and the M phase (mitotic phase), which composes of two major processes: mitosis, constituting the pairing and separation of duplicated chromosomes, and cytokinesis, whereby the cell splits into two daughter cells (Schafer, 1998). On completion of mitosis a cell either enters into G₁ phase and continues another cell cycle or ceases to divide, entering a quiescent or resting phase (G₀) from a cell cycle checkpoint in the G₁ phase, such as the restriction point (animal cells) or the start point (yeast). This usually occurs in response to a lack of growth factors or nutrients. Cells in G₀ may re-enter the cell cycle, start dividing and/or undergo a pathway leading to terminal differentiation (Schnerch et al., 2012). Regulation of cell cycle is a key mechanism for the maintenance of homeostasis of normal cell growth and viability and is tightly controlled by a series of regulatory molecules which include a group of regulatory subunits, the cyclins and a group of catalytic kinase subunits known
as cyclin-dependent protein kinases (Cdks). The kinase part of the complex is an enzyme that adds a phosphate to various proteins required for progression of a cell through the cell cycle. Different cyclins bind specifically to different Cdks to form distinct complexes at specific phases of the cell cycle, thereby, causing the cell to move from $G_1$ to $S$ or $G_2$ to $M$ phase in a timely and sequential manner (Li and Brooks, 1999; Vermeulen et al., 2003; Bloom and Cross, 2007). Thus, failure in any of the regulatory mechanisms may lead a cell to grow and divide in an uncontrolled manner and contribute to tumor development (Maya-Mendoza et al., 2009).

The term tumor is derived from the Latin word "tumere" meaning to swell. It is an abnormal mass of solid or fluid-filled tissue and may be classified as slowly growing ‘benign’ or rapidly growing ‘malignant’ forms (Vincent, 1985). Malignant tumors grow in a series of steps: the first is hyperplasia, having increase in the number of cells due to uncontrolled cell division. The second step is dysplasia, which results from further growth, accompanied by abnormal changes to the cells and the third step is anaplastic, with more abnormal cells that lose their original structure and function, often spreading over a wider area of tissue. At this stage, the tumors are non-invasive and non-lethal. The last step occurs when the cells in the tumor invade surrounding tissue, including the bloodstream, and are able to colonize other organs and the process is known as metastasis.

The terms neoplasm/neoplasia (new abnormal growth) and malignant tumor are commonly used interchangeably (Friedberg, 1986). Malignant tumors are commonly referred to as ‘cancer’ suggesting its tendency to cling and reach out to adjacent tissues (Abercrombie and Ambrose, 1962). The origin of the word ‘cancer’ is credited to the Greek physician Hippocrates. He used the terms “carcinos” and “carcinoma” (Greek-crab) to describe non-ulcer forming and ulcer-forming tumors because the finger-like spreading projections from a cancer looked like the shape of a crab. Cancer is today
recognized as a highly heterogeneous disease and a vast medical problem that has become the leading cause of human mortality (Gibbs, 2000). According to the cancer report released by World Health Organization (WHO) in 2003, cancer rates could further increase by 50% to 15 million new cases in the year 2020 (Stewart and Kleiheus, 2003). Studies also refer cancer as a "developmental disorder" (Dean, 1998) due to its involvement in the disruption of normal developmental program for cells, in terms of both differentiation and proliferation. Carcinogenesis is a multistage disease process by which normal cells are transformed into cancer cells. The events leading to cancer is sequential, involving both intrinsic and extrinsic factors. Cancer results from a series of molecular events that is viewed as a multistep process involving mutation and selection of cells with progressively increasing capacity for proliferation, survival, invasion, and metastasis. There are many risk factors for developing cancer. Substances/agents that induce cancer in both experimental animals and humans are called carcinogens. These include: radiation e.g. UV-rays, X-rays, gamma radiation, etc.; chemicals e.g. benzo(a)pyrene, aflatoxin, benzene, dimethylnitrosamine, etc.; and viruses such as Hepatitis B and C viruses, Epstein-Barr virus, Human T-cell leukemia virus type I and human papillomaviruses (Butel, 2000). For a cell to become cancerous, multiple mutations or changes in many genes are required which together allow the cell to escape normal control mechanisms (Croce, 2008) which usually result from mutations in genes that regulate cell division. Genes involved in tumorigenesis include those whose products- directly regulate cell proliferation (by either promoting or inhibiting), control programmed cell death or apoptosis, and are involved in the repair of damaged DNA. Two classes of regulatory genes- the oncogenes and tumor suppressor genes are directly involved in carcinogenesis (Vogelstein and Kinzler 1998; Bishop and Weinberg 1996). Molecular pathogenesis of cancer cells in various stages of progression may be due to activation of altered expression of proto-oncogenes to oncogenes and the loss or
inactivation of tumor suppressor genes whose normal function is to control cellular growth and differentiation, thus, correlating with the clinical aggressiveness of cancer (Fearon and Vogelstein, 1990; Yokota, 2000).

Proto-oncogenes encode proteins that are involved in the control of cell growth. Alteration in the structure and/or expression of proto-oncogenes can activate them to become oncogenes which are involved in the expression of malignancy (Todd and Wong, 1999). Three genetic mechanisms have been suggested to facilitate carcinogenesis, they are: mutation, gene amplification, and chromosomal rearrangements. (i) Mutations activate proto-oncogenes through structural alterations in their encoded proteins. These alterations involve critical protein regulatory regions which often lead to uncontrolled, continuous activity of the mutated protein. Various types of mutations, such as base substitutions, deletions, and insertions, are capable of activating proto-oncogenes; (ii) Gene amplification refers to the expansion in copy number of a gene or increase in amount of certain protein within the genome of a cell. The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogeneous staining regions (HSRs). Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth. The frequent observations of DMs and HSRs in human tumors suggest that amplification of specific proto-oncogenes may be a common occurrence in neoplasia. Some studies demonstrated that three proto-oncogene families- myc, erb B, and ras are amplified in a significant number of human tumors; (iii) chromosome rearrangements consist mainly of chromosomal translocations and, less frequently, chromosomal inversions. Chromosomal rearrangements can lead to hematologic malignancy via two different mechanisms- the transcriptional activation of proto-oncogenes or the creation of fusion genes. Transcriptional activation, sometimes referred to as gene activation, results from
chromosomal rearrangements that move a proto-oncogene close to an immunoglobulin or T-cell receptor gene which then falls under control of regulatory elements from the immunoglobulin or T-cell receptor locus. This circumstance causes deregulation of proto-oncogene expression, which can then lead to neoplastic transformation of the cell. This mechanism results in either an alteration of proto-oncogene structure or an increase in proto-oncogene expression. For example, the overexpression of RING1 results in enhanced expression of the proto-oncogenes c-jun and c-fos and leads to oncogenic transformation by deregulation of the expression levels of certain oncogenes (Satijn and Otte, 1999). It is also reported that aberrant c-myc expression can contribute to the development of neoplasia (Corcoran et al., 1984). Because neoplasia is a multistep process, more than one of these mechanisms often contributes to the genesis of cancers by altering a number of cancer-associated genes. Full expression of the neoplastic phenotype, including the capacity for metastasis, usually involves a combination of proto-oncogene activation and tumor suppressor gene loss or inactivation. For instance, activation of oncogene in normal cells includes proto-oncogenes that code for the proteins which send a signal to the nucleus to stimulate cell division. These signaling proteins act in a series of steps called signal transduction cascade or pathway. In each step of the pathway, one factor or protein activates the next. However, the altered versions of proto-oncogenes, i.e. the oncogenes activate the signaling cascade continuously, resulting in an increased production of factors that stimulate growth. ras is an oncogene that normally functions as an “on-off” switch in the signal transduction cascade. Mutations in ras cause the signalling pathway to remain “on,” leading to uncontrolled cell growth. About 30% of tumors- including lung, colon, thyroid, and pancreatic carcinomas have a mutation in ras.

Tumor suppressor genes normally act as cell’s brake by encoding proteins that inhibit cell growth, preventing tumor formation. Mutations in these genes result in a loss
of function of one or more tumor suppressor genes making them inactive, hence, cells no longer show normal inhibition of growth and division. Haber and Harlow (1997) proposed a novel description of tumor suppressor genes as “genes that sustain loss-of-function mutations in the development of cancer”. Thus, inhibition of tumor suppressor genes may contribute and promote uncontrolled cell growth. A tumor suppressor may possess multiple mechanisms to suppress cancer cell growth (Sherr, 2004). To date, four major mechanisms have been revealed for tumor suppressors. They are: suppression of cell division, induction of apoptosis, DNA damage repair and inhibition of metastasis.

Suppression of cell division is the main mechanism for most tumor suppressors. Some of the tumor suppressors that adopt this mechanism include Rb, adenomatosis polyposis coli (APC), alternate reading frame (ARF), p15, p16, p18, p19, p21, p27, and p53. Rb, which is the first discovered tumor suppressor, inhibits the transcription of specific genes required for mitosis through binding to transcription factors such as E2Fs, which are key cell proliferation regulators (Vandel et al., 2001). Tumor suppressor protein p53, which can also bind to DNA, stimulates the expression of other genes, such as WAF1/CIP1 encoding p21 (Vaziri et al., 2003). It is suggested that p53 gene mutations may be involved in colorectal neoplasia, through inactivation of a tumor suppressor function of the wild-type p53 gene (Baker et al., 1989). Apoptosis is another functional mechanism of tumor suppression. Examples of this group of tumor suppressors are p53, APC, cluster of differentiation 95 (CD95), bridging integrator 1 (Bin1) and phosphatase and tensin homolog (PTEN).

Apoptosis is regulated by many different pathways integrating both positive and negative regulations. p53 mediates apoptosis through two major pathways- the extrinsic pathway, which activates a caspase cascade, and the intrinsic pathway which promotes the apoptosome formation via the Bcl-2 family (Haupt et al., 2003). APC, which has been observed to be frequently mutated in colorectal cancer, promotes transcription-
independent apoptosis via caspase 8 (Steirgerwald et al., 2005). The tumor suppressors that can help in DNA damage repair include mutS homolog 2 (MSH2), mutL homolog 1 (MLH1), Ataxia-telangiectasia-mutated gene product (ATM), breast cancer protein (BRCA), Nijmegen breakage syndrome 1 (NBS1), Fanconi-Anemia–related tumor suppressor (FA), and p53 (Sherr, 2004). These tumor suppressors are able to fix DNA damages, including mismatch and vast damage to one of the DNA double strands. Generally, p53 can induce nucleotide excision repair (NER) to remove damaged DNA portions and mediate synthesis from the other strand; whereas MSH2 and MLH1 can repair DNA mismatch (Seifert and Reichrath, 2006). The majority of cancer death is caused by metastasis (Yoshida et al., 2000). During metastasis, tumor cells have signal interactions with endothelial cells to initiate angiogenesis and break down vascular walls that promote their spread. Tumor suppressors that can inhibit metastasis consist of metastin, breast cancer metastasis suppressor 1 (BRMS1), tissue inhibitor of metalloproteinase (TIMP), cofactor required for specificity protein 1 activation (CRSP), and KAL1/CD82. The binding of metastin to the metastin receptor (orphan G protein-coupled receptor GPR54) increases the expression and activity of focal adhesion kinase (FAK) and inhibits the metastasis of melanoma cells (Ohtaki et al., 2001).

Cancers are classified by the type of cell that was initially altered. The main categories include: carcinomas, sarcomas and leukemia/lymphoma (Cairns, 1986). Carcinomas are cancers of epithelial cells of the ectodermal or endodermal origin. This group includes many of the most common cancers, particularly in the aged, and includes nearly all those developing in skin and epithelial linings of internal organs and glands, breast, prostate, lung, pancreas, colon, etc. Carcinomas account for approximately 85% of human cancers. Sarcomas are those arising from connective tissue each of which develops from cells originating in mesenchymal cells (i.e. bone, cartilage, fat, nerve and muscle). Although they account for most of the cancers studied in laboratory animals,
they constitute only about 2-3% of human cancers. Lymphomas and leukaemia arise from hematopoietic cells that leave the bone marrow and tend to mature in the lymph nodes (Hodgkin's and non-Hodgkin's lymphoma) and blood respectively. They constitute about 10% of human cancers.

Hanahan and Weinberg (2000) proposed that malignant growth is a manifestation of six essential alterations in cell physiology as follows:

i. **Self-sufficiency in growth signals**- Normal cells require a variety of external growth signals (epidermal growth factors (EGF), platelet-derived growth factors (PDGF), fibroblast growth factor (FGF), etc.) to grow and divide while cancer cells can grow and divide without external growth signals. The production of these growth signals is tightly controlled in cellular environment while cancer cells develop numerous ways to generate their own growth signals like some oncogene products that act by mimicking normal growth signaling and disrupts the homeostatic mechanism of normal cells.

ii. **Insensitivity to growth-inhibitory (anti-growth) signals**- Multiple anti-growth signals operate within a normal tissue and act on the cell cycle clock, by interrupting cell division in the interphase. However, cancer cells become insensitive to these anti-growth signals. For example, the growth inhibitor signals are funneled through the downstream pRb, the product of Rb tumor suppressor gene, which prevents the inappropriate transition from G1 to S phase. If pRb is damaged or disrupted through a mutation in its gene, the cell can divide uncontrollably and may lead to cancer formation (Weinberg, 1995; Nevins, 2001).

iii. **Evasion of programmed cell death (apoptosis)**- Evasion of apoptosis contributes to both tumorigenesis and treatment resistance (Fulda, 2009; Fernald and Kurokawa, 2013). Cell death pathways can be blocked at different levels of the signaling cascade by up-regulation of anti-apoptotic proteins (Bcl-2, Bcl-XL, and Mcl-1).
and/or by downregulation or dysfunction of pro-apoptotic molecules such as Bax, Bak and BH3 domain (Adams and Cory, 2007).

iv. **Limitless replicative potential**- Telomerase activity is elevated in vast majority of tumors while it is tightly repressed in normal cells. The telomerase activation has been observed in ~90% of human cancers (Shay and Bachhetti, 1997). Although the mechanisms are not completely understood, in tumor cells, the telomerase enzyme prevents the formation of critically short telomeres, adding GGTTAG repeats to the end of the chromosomes immortalizing the cells (Tarkanyi and Aradi, 2007; Kelland, 2007).

v. **Sustained angiogenesis**- By the process of angiogenesis, tumor cells make growth factors which induce formation of new capillary blood vessels to supply the cells with nutrients and oxygen. For sustaining tumor growth, two proteins appear to be most important- the vascular endothelial growth factors (VEGF) and basic fibroblast growth factor (bFGF) (Zetter, 1998).

vi. **Tissue invasion and metastasis**- Malignant cells invade adjacent tissues (invasion) and then travel to distant sites and establish metastasis. About 90% of human cancer deaths results from metastatic spread of the primary lesions (Sporn, 1996). Several classes of proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell-cell adhesion molecules such as cadherins and integrins (Aplin et al., 1998) and matrix degrading proteases (Chambers and Matrisian, 1997).

**Cell surface in malignancy**: Changes in the cell surface may be an important element in the differences between normal and malignant cells. Cell surface components could play a vital role in the malignant transformation of a variety of cells. Cancer cells are reported to have shown high agglutination property with lectins while normal cells do not agglutinate appreciably (Prasad and Sodhi, 1981). Prasad (1986) reported that the agglutination behaviour of normal and malignant cells depends upon the sialic acid
moieties present on the cell surface. Sialic acid is an important biological tumor marker of high sensitivity and specificity in diagnosis and response to treatment of cancer (Stringou et al., 1992; Chang et al., 2009). With the progress of malignancy and metastasis, the cell surface glycoproteins and glycolipids (gangliosides) show marked elevations (Stringou et al., 1992; Gokmen et al., 2004). Sialic acid molecules are also involved in cell communication such as cell-cell and cell-matrix interactions and molecular recognition during tumor development, differentiation and progression (Schaur, 1985) which is catalyzed by enzymes of the sialyltransferase family (Wang, 2005). It has been suggested that tumor cells have the ability to change their surface properties and alter the sialo-glycoconjugates expressed on their plasma membranes which affect their behaviour and ability to invade (Passaniti and Hart, 1988). Since, sialic acids possess relatively strong carboxyl groups (N-acetyl neuraminic acid), their presence in glycoproteins or at the cell periphery makes a significant contribution to the negative surface charge (Schaur, 1985), and are responsible for the electrostatic repulsion as seen with platelets, erythrocytes and carcinoma cells. Although elevated levels of sialic acid have been associated with malignancy (Kokoqlu et al., 1992; Michalakis et al., 2012; Taqi, 2012), however, the sialic acid level could be decreased therapeutically (Nicol and Prasad, 2002; Celen et al., 2006; Yadav et al., 2011). Thus, evaluation of sialic acid changes could be very helpful by contributing both the diagnosis of patients and to monitoring their progression and response to treatment.

**Apoptosis and cancer:** Apoptosis is a genetically regulated mechanism of cell turnover that occurs during embryonic development, normal cellular homeostasis, and spontaneous and drug-induced tumor cell death. It is a tightly regulated multi-step pathway that is responsible for cell death not only during development but also in adult multicellular organisms in which it partly controls cell numbers. Kerr et al. (1972) described a distinct morphology of dying cells and called it apoptosis. The term was
coined based on the fact that the release of apoptotic bodies by dying cells resemble the picture of falling leaves from deciduous trees, called “apoptosis” in greek (Bjelakovic et al., 2005). This type of cell death has also been called cell-suicide or programmed cell death, and is characterized by a genetic controlled autodigestion of the cell through the activation of endogenous proteases containing cysteine residues collectively termed as "caspases". This process results in cytoskeletal disruption, cell shrinkage, membrane blebbing, loss of cell-cell contact, chromatin condensation and internucleosomal DNA fragmentation which form the apoptotic bodies that are then engulfed by neighbouring phagocytic cell (Erwig and Henson, 2008; Elliot and Ravichandran, 2010). Being a gene controlled process apoptosis is susceptible to disruption by mutations (Lowe and Lin, 2000). Hence, it soon became clear that failure to undergo apoptosis might be involved in the pathogenesis of a variety of human diseases (Thompson, 1995; Chamond et al., 1999) such as viral infections, autoimmune diseases, and cancer (Kasibhatla and Tseng, 2011).

1.2 CANCER THERAPY

The goals of cancer treatments are to cure cancer by killing or removing all cancer cells, prevent or delay the cancer from coming back. Often, it requires the artful combination of more than one type of cancer therapy. The choice of therapy depends upon the location and grade of the cancer and the stage of the disease, as well as the general state of the patient. Various methods have been developed to treat cancer, some of which are:

**Surgery**- It is the removal of the tumor and surrounding tissue during an operation and is the primary treatment for many types of cancer. Some cancers can be completely removed with surgery alone, but undetected malignant cells may metastasize to other organs. Surgery may also be used to confirm a diagnosis (such as with a surgical biopsy), determine the extent of the cancer (called staging), and relieve side
Effects (such as removing an obstruction to ease pain). Some cancers can be treated surgically with less-invasive techniques, such as laser surgery which uses a powerful beam of high-energy light to vaporize certain tumors of the cervix, larynx, and skin. Examples of surgical procedures for cancer include mastectomy for breast cancer, prostatectomy for prostate cancer and lung cancer for non-small cell lung cancer.

**Radiation therapy**- Also called radiotherapy, x-ray therapy, or irradiation therapy uses ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be given externally via external beam radiotherapy (EBRT) which is radiation given from a machine outside the body or internally via brachytherapy when radiation treatment is given using implants. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to grow and divide continuously. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions. Radiation therapy may be used to treat almost every type of solid tumor including cancers of the brain, breast, cervix, larynx, liver, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma. Radiation dose to each site depends on a number of factors including the radiosensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by radiation. Thus, as with every form of treatment, radiation therapy is not without its side effects.

**Targeted therapy**- Targeted therapies make use of therapeutic antibodies or small molecules allowing treatment to be more tumor-specific and less toxic. These small molecules/peptides can bind to cell surface receptors or affected extracellular
matrix surrounding the tumor (Wu et al., 2006). Radionuclides which are attached to these peptides (e.g. RGDs) eventually kill the cancer cell if the nuclide decays in the vicinity of the cell. This treatment targets the cancer’s specific genes, proteins, or the tissue environment that contributes to cancer growth and survival. Thus, blocking the growth and spread of cancer cells while limiting damages to normal cells, may help cancer medications.

**Immunotherapy**- Immunotherapy is designed to boost the body’s natural defenses to fight cancer and is also called biological therapy. Contemporary methods for generating an immune response against tumors include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines to generate specific immune responses are the subject of intensive research for a number of tumors, notably malignant melanoma and renal cell carcinoma. The side effects of immunotherapy generally include flu-like symptoms, such as chills, nausea, and fever.

**Hormonal therapy**- In this therapy, cancers usually of the prostate, breast, thyroid, and reproductive system is treated by lowering the amount of hormones in the body (Chen, 2009; Miura et al., 2011). Removing or blocking estrogen or testosterone is often an important additional treatment. For example, the production of testosterone is reduced considerably in men with prostate cancer. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

**Small molecule sequential dual targeting theragnostic strategy (SMSDTTS):** SMSDTTS is an unconventional recent anticancer approach that aims to target and debulk the tumor mass, wipe out the residual tumor cells, meanwhile enabling cancer detectability (Li et al., 2011). This dual targeting approach works in two steps for systemic delivery of two naturally derived drugs- in the first step, an anti-tubulin
vascular disrupting agent (eg. combretastatin A4 phosphate (CA4P) is injected, to selectively cut off tumor blood supply and to cause massive necrosis, which nevertheless always leaves peripheral tumor residues, and second step requires administration of a necrosis-avid radiopharmaceutical, namely $^{131}$I-hypericin ($^{131}$I-Hyp), on the next day, which accumulates in intratumoral necrosis and irradiates the residual cancer cells with beta particles thereby preventing tumor relapse. Studies on the possible drawbacks, practical challenges and future improvement with SMSDTTS has also been discussed for further preclinical to clinical applications (Li et al., 2013).

**Chemotherapy**- It is the use of drugs to kill cancer cells, usually by stopping the cancer cells’ ability to grow and divide and is used either singly as monotherapy or in combination with surgery and/or radiotherapy (Takimoto and Calvo, 2009). In chemotherapy, hundreds of drugs like cisplatin (CDDP), carboplatin, cyclophosphamide, doxorubicin, melphalan, mitomycin C, gemcitabine, etc. have been approved for use against various types of cancer (Black and Livingston, 1990a, b). Almost all anticancer drugs work by affecting DNA synthesis; they do not kill resting cells unless those cells divide soon after exposure to the drug. These drugs interfere with cell division in various possible ways, e.g. CDDP and carboplatin complexes inhibit DNA synthesis through covalent binding of DNA molecules to form intrastrand and interstrand DNA crosslinks; cyclophosphamide, gemcitabine and melphalan compounds crosslink DNA by binding at N7 position of guanine and induces inhibition of DNA synthesis, leading to cell death; doxorubicin has been shown to inhibit DNA topoisomerase II which is critical to DNA function. However, therapeutic efficacy of most of these drugs are limited due to the development of various side effects such as nephrotoxicity by CDDP (Yao et al., 2007), myelodysplastic syndrome (MDS) and gonadal toxicity after cyclophosphamide administration (Haubitz, 2007), doxorubicin-induced acute cardiac injury and chronic congestive heart failure (Arola et al., 2000)
and/or the acquired drug resistance by cancer cells (Kartalou and Essigmann, 2001; Lippert et al., 2008). Conventional cytotoxic anticancer chemotherapeutic drugs were also developed with the intent of treating cancer by direct killing, or inhibition of growth of cycling tumor cells. Hence, there has been considerable interest in the notion of exploiting such drugs as angiogenesis inhibitors (Miller et al., 2001; Gately and Kerbel, 2001; Kerbel and Folkman, 2002).

1.3 CDDP AS A CANCER CHEMOTHERAPEUTIC AGENT

Platinum-based complexes are important drugs that represent a unique class of DNA-damaging antitumor agents (Kostova, 2006). The compound platinum occurs in two isomers: transplatin and cisplatin (CDDP). The two compounds have the Pt(II) in a square planar coordination. Kurnakow, in 1894 developed a method for distinguishing the geometry of the two complexes by reacting with thiourea. Cleare and Hoeschele (1973) summarized the structure-activity relationships for a class of platinum coordination compounds and confirmed that only those compounds having cis geometry of the leaving group is necessary for antitumor activity. The most active complex, CDDP have the chloride and ammonia moieties in cis position and was therefore, found to exhibit the antitumor activity, whereas, its trans isomer showed no such activity.

CDDP (cis-diamminedichloroplatinum (II) or cisplatin) is one of the most widely used antineoplastic drugs of choice in many platinum based chemotherapy regimens. It acts by damaging DNA owing to platination to form covalent platinum DNA adducts (Jamieson and Lippard, 1999; Boulikas and Vougiouka, 2003). The synthesis and characterization of CDDP was first reported by Michele Peyrone in 1845 (Kauffman, 2010), and had known for a long time as Peyrone’s salt. However, it was not until 1893, that Alfred Werner correctly proposed its square planar configuration and distinguished
between the cis and trans isomers- CDDP and transplatin (Figure 1) for which he won the Nobel Prize for Chemistry in 1913 (http://www.nobel.se).

![Cisplatin (CDDP) and Transplatin](image)

**Figure 1**: The chemical structures of two isomeric platinum complexes- cisplatin (CDDP) and transplatin.

In 1965, Barnett Rosenberg and co-workers at Michigan state university discovered that electrolysis of platinum electrodes generated a soluble platinum complex which inhibited binary fission in *Escherichia coli* bacteria. Although bacterial cell division was arrested, cell growth continued and the bacteria grew as filaments up to 300 times their normal length (Rosenberg et al., 1965). The octahedral Pt(IV) complex cis PtCl$_4$(NH$_3$)$_2$, but not the trans isomer, was found to be effective at forcing filamentous growth of *Escherichia coli* cells. The square planar Pt(II) complex cis PtCl$_2$(NH$_3$)$_2$ turned out to be even more effective at forcing filamentous growth. This finding led to the observation that cis PtCl$_2$(NH$_3$)$_2$ was indeed highly effective at regressing the mass of sarcomas in rats. Confirmation of this discovery and extension of testing to other tumour cell lines launched the medicinal applications of CDDP (Rosenberg et al., 1967).

Based on these results, further clinical trials of CDDP increased rapidly that showed a high level and broad spectrum of antitumor activity against experimental tumors such as Dunning ascitic leukemia and intramuscular Walker 256 carcinosarcoma in rats, which resulted in pronounced regression (Kociba et al., 1970). CDDP was also shown to be highly effective in promoting regression of rat DMBA mammary carcinoma (Welsh, 1971), and active against tumors that are normally drug-resistant (eg.
L1210 murine leukemia) and virally induced (e.g. Raou Sarcoma) as reviewed and summarized by Rosenberg (1985). Additional reports of clinical trials have also shown CDDP to have high degree of anticancer activity against testicular cancer (Higby et al., 2006) and ovarian cancer (Wiltshaw and Carr, 1974). CDDP was approved by the Food and Drug Administration (FDA) in 1978, and the cure rate for testicular cancer after CDDP treatment, when promptly diagnosed is now noted to be greater than 90% (Bosl and Motzer, 1997). CDDP is also used to treat other types of human malignancies including bladder (Griffiths et al., 2011), cervical (Lukka et al., 2002), head and neck cancer (Qin et al., 2012) and non-small cell lung cancer (Johnson, 2000).

CDDP is a water soluble, square planar coordination compound containing a central platinum atom surrounded by two chloride atoms and two ammonia moieties in the cis-position (Figure 1). The molecular formula of CDDP is PtCl$_2$H$_6$N$_2$, and its molecular weight is 300.1. It remains stable under normal temperatures and pressures, but may transform slowly over time to the trans-isomer (IARC 1981, Akron 2009). CDDP is slightly soluble in water and soluble in saline at 1 mg/ml and in N,N-dimethylformamide. It is used for the treatment of various malignancies, often in combination with other antineoplastic agents (IARC 1981, HSDB 2009) and is available as injectable solutions at a concentration of 1mg/ml (http://www.ntp.niehs.nih.gov/ntp/roc/twelfth/profiles/Cisplatin.pdf#search=cisplatin.)

### 1.3.1 Biochemical mechanisms of action of CDDP

The biochemical mechanism by which CDDP passes the cell membrane still remains unclear though initially it was suggested that passive diffusion is the main mechanism by which CDDP enters the cell. In 1981, Byfield and Calabro-Jones first proposed that CDDP could be transported actively via the carrier-mediated transport. Several transporters, including the Na$^+$K$^+$-ATPase and others have been implicated in
facilitating the entry of CDDP into the cells (Hall et al., 2008). Moreover, recent observations also reveal a direct connection between the cellular concentrations of copper and platinum (Nitiss, 2002; Sinani et al., 2007). The plasma membrane copper transporter-1 (CTR1) showed a defect in Ctr1 gene that decreased CDDP accumulation in yeast (Ishida et al., 2002; Safaei and Howell, 2005). Thus, CDDP drug probably enters the cell by a number of active influx transporters along with passive diffusion through cell membrane and consequently, one or both chloride ligands is slowly displaced by water in a process termed as aquation resulting in the formation of highly charged platinum complex- \([\text{PtCl}(\text{H}_2\text{O})(\text{NH}_3)_2]^+\). On further hydrolysis, the other chloride ligand is also displaced.

**Drug accumulation inside cells and binding to non-DNA targets:** Following intravenous (i.v.) administration of CDDP in the host, the drug rapidly diffuses into the tissues and highly binds to plasma proteins resulting in a strong reactivity of platinum against the thiol groups of amino acids. In this way, about 90% of the platinum in the blood is bound to albumin and other plasma proteins leading to inactivation of a large amount of CDDP molecules. In addition, before CDDP accumulates in the cell, it may bind to phospholipids and phosphatidylserine of the cell membrane (Speelmans et al., 1997). In the cytoplasm, many cellular constituents that have soft nucleophilic sites such as cytoskeletal microfilaments, thiol containing peptides, proteins and RNA react with CDDP (Jordan and Carmo-Fonseca, 2000). Hence, only 5-10% of covalently bound cell-associated CDDP is found in the DNA fraction, whereas, 75-85% of the drug binds to proteins and other cellular constituents. The most important non-DNA target of CDDP is probably the tripeptide glutathione (GSH) which is present in cells at high concentrations. GSH and other thiol- containing biomolecules such as metallothioneins (MT) have been reported to bind rapidly to platinum and this binding has primarily been
associated with negative phenomena, including the development of resistance and toxicity (Hrubisko et al. 1993).

**Binding to DNA:** It is accepted that binding of CDDP to nuclear or genomic DNA in the cell is largely responsible for its anticancer effects (Gonzalez et al., 2001). The ability of CDDP to react with DNA and formation of CDDP-DNA adducts with inter- and intrastrand nuclear DNA crosslink is suggested to be the main mechanism underlying its cytotoxic effect that lead to the death of cancer cells (Florea and Busselberg, 2011). The formation of CDDP-DNA adducts may then block replication and/or prevent transcription (Payet et al., 1993; Zhu et al., 2012). As compared to nuclear DNA (nDNA), CDDP has also been shown to bind preferentially to mitochondrial DNA (mtDNA) (Yang et al., 2006; Podratz et al., 2011) where CDDP adduction is reported to be 4 to 8 fold higher (Olivero et al., 1995). This preferential binding of CDDP may be explained by the naked structure of mtDNA, which makes it highly accessible to damaging agents. However, for interaction to occur with DNA, the neutral CDDP has to be activated through a series of spontaneous aquation reactions, which involve the sequential replacement of the cis-chloro ligands of CDDP with water molecules (Kelland, 2000).

CDDP binds more tightly with nitrogen (N) because it balances the platinum charge more effectively than chlorine. Hence, the molecule loses its chlorine atoms in exchange for the N-atom of the target guanines. The N7 atoms of the imidazole rings of guanine and adenine located in the major groove of the double helix are the most accessible and reactive nucleophilic sites for platinum binding to DNA. Thus, in the nucleus, CDDP primarily interacts with the N7-sites of purine residues in the DNA to form DNA-DNA inter- and intrastrand crosslinks (Kartalou and Essigmann, 2001; Trzaska, 2005). The intrastrand crosslinks form adducts with two consecutive guanine bases within a strand of DNA. It is noted that these 1,2- intrastrand adducts, ApG and
GpG are the major forms of the DNA adducts responsible for the cytotoxic effects of CDDP and account for about 80-90% of total adducts (Cepeda et al., 2007), while minor adducts that include the interstrand crosslinks and 1,3-intrastrand crosslinks account for only a few percent (Figure 2).

**Figure 2**: Main adducts formed after binding of cis-DDP to DNA. (A) 1,2-intrastrand cross-link, (B) interstrand crosslink, (C) monofunctional adduct, and (D) protein-DNA crosslink. The main site of attack of cis-DDP to DNA (N7 of guanine) is shown in the central panel (Source: Cepeda et al., 2007).

DNA adduct formation is followed by DNA damage recognition by over 20 proteins including hMSH2 of the mismatch repair (MMR) complex, the high-mobility groups (HMG1 and 2) proteins and the transcriptional factor “TATA-binding protein” (TBP). The recognition of 1,2-intrastrand adduct by these proteins may be the first step towards the initiation of apoptosis or programmed cell death. It is these adduct induced DNA bands that allow the binding of proteins such as the high mobility group (HMG)-domain between adjacent guanines (Pil and Lippard, 1992; Pasheva et al., 2002). The HMG proteins bind to DNA-CDDP adducts with high affinity and specificity (Zlatanova et al., 1998; Ohndorf et al., 1999). Once the protein is bound to the DNA, it inserts the phenyl group, phenylalanine into the minor groove created by the band. The
tightly bound HMG proteins then cause destacking of nucleotide bases resulting in DNA helix becoming kinked. With the HMG proteins bound to the DNA, the modified strand is not repaired properly and so the cytotoxic processes eventually end up in cell death.

Structural differences between the complexes formed by CDDP may serve as a molecular basis for their differential biological and chemotherapeutical activity. Several mechanisms suggest HMG proteins could protect CDDP-DNA adducts from recognition by DNA repair enzymes (Brown et al., 1993; Zamble et al., 1996; Arioka et al., 1999). CDDP is very effective in the treatment of testicular cancer and several HMG-domain proteins are specifically expressed in the testis and could potentially contribute to the CDDP sensitivity of testicular tumors. Although interstrand crosslinks represent a small amount of total CDDP lesions, it is uniquely recognized by nuclear proteins and strongly inhibit transcription in live mammalian cells to an extent not less than that of intrastrand crosslinks and thus play a role in its pharmacological activity (Zhu et al., 2013).

The CDDP-DNA adducts are subject to repair by several types of proteins, including the nucleotide excision repair (NER) proteins, mismatch repair (MMR) proteins and DNA-dependent protein kinase (DNA-PK).

**NER proteins**: NER is a major pathway used by human cells for the removal of damaged or inappropriate bases from DNA. It is an ATP-dependent multiprotein complex that primarily repairs via the NER system by recognising the bending induced on DNA by the 1,2- intrastrand crosslinks, and subsequently excises the part of the DNA that includes the kink as 27- to 29-base-pair oligonucleotides. The gap that remains is then filled by DNA polymerase (Chaney and Sancar, 1996). The favorable response of testicular cancer to CDDP chemotherapy was associated with low levels of NER proteins XP complementation group A (XPA) and excision repair cross-
complementation group I (ERCCI) (Welsh et al., 2004). Functional NER was also required for CDDP-induced transcription of Bcl-xL via nuclear factor-kappa B (NF-κB) (Lomonaco et al., 2009).

**MMR proteins:** MMR is a post-replication repair system that corrects replication error (Modrich, 1997). It has been proposed that MMR proteins would try to insert the “correct” nucleotide on the non-damaged strand opposite to the intrastrand adduct between the adjacent guanines which then induces cell death pathways. MMR pathways play an important role in modulating CDDP cytotoxicity (Kothandapani et al., 2013). It has been reported that the interaction between MMR protein MLH1/post-meiotic segregation 2 (PMS2) and p73 enhances DNA damaged-induced apoptosis (Shimodaira et al., 2003).

**DNA-PK:** It is a heterotrimeric protein that includes Ku70/Ku80 heterodimer and a catalytic subunit, DNA-PK. This complex is required for the elimination/repair of DNA double-strand breaks (DSB) that are induced by ionizing radiation. DNA-PK associates with DNA ends via interactions with the Ku DNA binding subunits in addition to direct interactions with DNA. Turchi and Henkels (1996) reported interaction of DNA-PK with CDDP-DNA lesions. Binding to Ku subunits of DNA-PK is essential *in vitro* to activate the kinase activity of DNA-PK for phosphorylation. It has been shown in apoptotic ovarian cancer cells that the presence of CDDP-DNA adducts serves to inhibit the ability of the Ku subunits of DNA-PK to translocate on a duplex DNA substrate so that kinase activity is revoked decreasing the ability of Ku subunits to bind DNA.

**CDDP induced cell death pathways:** CDDP is a very potent inducer of apoptosis (Henkels and Turchi, 1997; Galluzzi et al., 2012). At least two distinct pathways have been proposed to contribute to CDDP-induced apoptosis *in vitro*. They are mediated by: (i) the tumor suppressor protein p53, and (ii) by the p53-related protein
p73. Coupling CDDP damage to apoptosis requires mismatch repair activity, and recent observations further suggest an involvement of the homologous recombinatorial repair system (Jordan and Carmo-Fonseca, 2000). It is generally accepted that failure to repair CDDP-induced DNA damage may finally result in the triggering of apoptosis. Apoptosis or “programmed cell death is considered a controlled pathway that is dependent on ATP (adenosine triphosphate) and on active protein synthesis. Experimental evidences also reveal that protein damage caused by CDDP rather than DNA damage plays a role in triggering apoptotic pathways (Gonzalez et al., 2001; Wang et al., 2004). Some types of cancer cells when exposed to CDDP have shown apoptotic features like blebbing of the cell membrane and cell shrinkage. In cell lines that are particularly resistant to CDDP, the drug produces characteristic features of necrosis or accidental cell death due to general cellular machinery failure (Lau, 1999; Sun et al., 2012). It has been found that mitochondria play a central role in apoptosis. CDDP induces apoptosis via activation of mitochondrial signalling pathways (Park et al., 2002). A general property of execution by apoptosis is the specific degradation of a series of proteins by the cysteine aspartate-specific proteinases (caspases) which are activated when an apoptotic stimulus induces the release of cytochrome c from mitochondria. CDDP-DNA damage induces a fall in the mitochondrial permeability transition (MPT) releasing factors such as reactive oxygen species (ROS), Bax, and Ca^{2+} that facilitate the rupture of mitochondria. This rupture releases cytochrome C and procaspase-9 that binds to cytosolic Apaf-1 and ATP in an apoptosome complex, leading to the activation of caspase-9 which subsequently activates caspase-3, 6, and 7 systems, finally resulting in chromosomal DNA fragmentation and cellular changes characteristic of apoptosis.

In addition to the interaction of CDDP with cellular DNA, changes in various biochemical/enzymatic parameters, immune response, cell surface structure have also
been observed which have led to propose the involvement of multistep and multilevel effects of CDDP in the tumor cells/host. Prasad et al. (1999) reported a decrease in the activities of enzymes such as Na\(^+\)K\(^-\)-ATPase, 5\(^n\)-nucleotidase, arginase and lactate dehydrogenase (LDH) in tumor cells and tissues of tumor bearing mice after CDDP treatment. Sodhi and Prasad (1985) reported differential effect of CDDP on the lectin, concanavalin A (Con A) and wheat germ agglutinin (WGA), agglutinability of splenocytes and Dalton’s lymphoma (DL) cells. The agglutinability of normal cells (splenocytes) increased and DL (tumor) cells decreased after CDDP treatment. Sodhi (1976) reported the depolymerization of microfilaments and formation of giant multinucleated cells after CDDP treatment. Nicol and Prasad (2002) reported that cyclophosphamide treatment of tumor-bearing mice resulted in an overall decrease of sialic acid contents in the DL cells as well as other tissues such as liver, kidney and testes which may help in tumor regression. Based on the various findings on the effect of CDDP in the cells particularly involving the cellular components other than DNA, it has been suggested that these may play an additional significant role in the anticancer activity of CDDP.

### 1.3.2 CDDP-induced toxicities

Despite its excellent anticancer activity, the therapeutic efficacy of CDDP is often hampered by the development of various side effects in the host (Siddik, 2003; Barabas et al., 2008). Some of the severe side effects that limit the clinical applications of CDDP include:

**Nephrotoxicity:** The main dose limiting side effects of CDDP is kidney damage. Renal toxicity increases with the dose and frequency of administration and cumulative dose of CDDP (Madias and Harrington, 1978). In patients, CDDP-induced nephrotoxicity manifests acutely and/or chronically. Biochemically, it is manifested by increasing creatinine values, decreased clearance and electrolyte wasting. Adequate
hydration and diuresis is used to prevent renal damage. Reports of accidental overdoses, all of which have led to renal failure, confirm the potency of CDDP as a renal toxin in humans (Chu et al, 1993). Clinically, CDDP nephrotoxicity develops after 10 days of drug administration and is manifested as lower glomerular filtration rate, higher serum creatinine, and reduced serum magnesium and potassium levels (Pabla and Dong, 2008). CDDP is toxic to renal proximal tubules and the molecular mechanism by which CDDP kills the cells is clearly reported by Hanigan and Devarajan (2003). CDDP has multiple intracellular effects which include regulating genes, causing direct cytotoxicity with ROS, activating mitogen-activated protein kinases, inducing apoptosis, and stimulating inflammation and fibrogenesis. These events cause tubular damage and tubular dysfunction with sodium, potassium, and magnesium wasting (Yao et al., 2007). The most serious and one of the more common presentation is acute kidney injury (AKI) which occurs in 20-30% of patients (Pabla and Dong, 2008; Miller et al., 2010).

Inhibition of PKCδ enhanced CDDP-induced cell death in multiple cancer cell lines, while protecting kidneys from nephrotoxicity by attenuating kidney cell apoptosis and tissue damage (Pabla et al., 2011).

**Ototoxicity:** It is the functional impairment and cellular degeneration of the tissues of the inner ear caused by therapeutic agents. CDDP treatment causes a hearing loss, which can also lead to deafness and occurs in a large percentage of patients when treated with higher dose (Rademaker-Lakhai et al., 2006). Hearing loss following CDDP chemotherapy appears to be related to dose, age of the patient, and other factors, such as noise exposure, exposure to other ototoxic drugs, depleted nutritional state, including low serum albumin and anaemia (Rybak et al., 2009). The mechanisms involve the production of ROS and/or depletion of scavenging enzymes which can trigger cell death (Rybak and Rajkumar, 2007). In animal studies and in human temporal bone investigations, several areas of the cochlea are damaged, including outer
hair cells in the basal turn, spiral ganglion cells and the stria vascularis, resulting in hearing impairment. As opposed to a 5% risk in children between 15 and 20 years of age, the incidence of ototoxicity is reported to be greater than 40% in children below 5 years of age (Li et al., 2004).

**Neurotoxicity:** It is a well-known dose limiting side effect of CDDP treatment, inducing mainly sensory neuropathy of the upper and lower extremities (Amptoulach and Tsavaris, 2011). Mechanism of platinum neurotoxicity remains unclear although it may involve platinum accumulation within the dorsal root ganglia (DRG) leading to atrophy or loss of peripheral sensory neurons (Liu et al., 2009). CDDP neurotoxicity is first characterized by painful paresthesias and numbness which typically occurs during the first few drug cycles. Loss of vibration sense, paraesthesia (abnormal sensation) and ataxia (uncontrolled body movement) can become apparent after several treatment cycles (McWhinney et al., 2009). Most patients treated with CDDP develop a symptomatic and clinically detectable sensory neuropathy, caused by its preferential uptake in the dorsal root ganglia which produces a dose-related large fibre sensory neuropathy (neuronopathy) (Meijer et al., 1999; Albers et al., 2011). Chronic application of CDDP accelerated accumulation of unrepaired intrastrand crosslinks in neuronal cells of mice with dysfunctional nucleotide excision repair (NER) (Dzagnidze et al., 2007).

**Hepatotoxicity:** Liver toxicity is also a major dose limiting side effect in CDDP-based chemotherapy. When CDDP is administered at high doses, hepatotoxicity can also occur (Cersosimo, 1993). Oxidative stress appears to play an important role in CDDP-induced hepatotoxicity. Administration of selenium and high dose of vitamin E protects liver against CDDP-induced oxidative damage (Naziroglu et al., 2004). Heme oxygenase (HO) and catalase are important protective responses against CDDP toxicity in the livers of tumor-bearing mice (Christova et al., 2003).
1.3.3 Cellular resistance to CDDP

It is generally known that treatment of CDDP may also be hampered due to the development of resistance by cancer cells to the drug. Resistance to CDDP can be either acquired to chronic drug exposure or present itself intrinsic to cells (Siddik, 2003). Some tissues are inherently resistant to the drug and remain unresponsive while malignancies such as ovarian cancer may respond to CDDP treatment initially but can acquire resistance to the drug over time (Jamieson and Lippard, 1999).

Resistance of cells to CDDP is multifactorial (Sedletska et al., 2005; Cepeda et al., 2007) involving several mechanisms (Figure 3) being encountered simultaneously within the same tumor cell (Galluzzi et al., 2012). The main activities that modulate the resistance to this drug are: (i) intracellular changes in drug accumulation, (ii) increased production of intracellular thiols to modulate toxicity, (iii) increased capability of cells to repair platinum-DNA adducts, and (iv) failure of cell death pathways (Kartalou and Essigmann, 2001; Rabik and Dolan, 2007). These resistance mechanisms are cell line-dependent so that a particular tumor may exhibit one, two or even all the above mentioned mechanisms. Altered expressions of oncogenes that subsequently limit the formation of CDDP-DNA adducts and activate anti-apoptotic pathways may also contribute as an additional pathway to the resistance phenotype.

GSH plays an important role in a multitude of cellular processes including cell differentiation, proliferation, and apoptosis. It has also become the focus of interest in cancer chemotherapy and has shown to be implicated in the metabolism of CDDP causing alterations in the rate of drug uptake. In the cells, under normal physiological conditions more than 98% of GSH exists in reduced form and is involved in a variety of important physiological and metabolic functions including synthesis of proteins, DNA transport, enzyme activity and protection of cells (Wang and Ballatori, 1998; Dringen, 2000).
Elevated GSH levels are observed in various types of tumors, and this makes the neoplastic tissues more resistant to chemotherapy. Intracellular GSH levels play an important role in regulating CDDP resistance (Kartalou and Essigmann, 2001; Rabik et al., 2007). A major function of GSH is the detoxification of xenobiotics and some endogenous compounds (Stewart, 2007). GSH inhibits free radical mediated injury by eliminating toxic peroxides and protects protein sulfhydryl groups from oxidation.

Three principle mechanisms have been proposed for the role of GSH in regulating CDDP sensitivities: (i) GSH may serve as a cofactor in facilitating multidrug resistance protein 2- (MRP2-) mediated CDDP efflux in mammalian cells, since MRP2-transfected cells were shown to confer CDDP resistance; (ii) GSH may serve as a redox-regulating cytoprotector based on the observations that many CDDP-resistant cells overexpress...
GSH and γ-glutamylcysteine synthesis (γ-GCS), the rate-limiting enzyme for GSH biosynthesis; (iii) GSH may also function as a copper (Cu) chelator. Elevated GSH expression depletes the cellular bioavailable Cu contents, resulting in upregulation of the high-affinity Cu transporter (hCtr1) expression which is also a CDDP transporter (Chen et al., 2008).

Several studies reported the level of drug accumulation in different cell lines having acquired CDDP resistance which include human ovarian carcinoma (Loh et al., 1992), human head and neck squamous cell carcinoma (Zhang et al., 2006a), human non-small cell lung carcinoma (Barr et al., 2013), human small cell lung cancer (Moritaka et al., 1998) and murine leukemia L1210 (Segal-Bendirdjian and Jacquemin-Sablon, 1995). Altered regulation of active transporters is responsible for reduced accumulation of CDDP in resistant cells (Hall et al., 2008). It is reported that loss of p53 or DNA mismatch repair function increases the rate of development of resistance to CDDP in human colon carcinoma cells (Lin and Howell, 2006).

An alternative way to circumvent CDDP resistance and to enhance its anticancer activity is the use of biochemical modulation strategies which involves the combination therapies of CDDP with a number of anticancer drugs or agents that interfere with specific CDDP-resistance pathways (Fuertes et al., 2003) and producing synergistic or at least additive effects in killing cancer cells, yielding a substantial improvement in the treatment of animal tumors (Bae et al., 2007; Bai et al., 2009) and human malignancies (Tsavaris et al., 1994; Anderson et al., 2004; Coxon et al., 2012) as well. CDDP in combination with other chemotherapeutic agents, such as etoposide, mitomycin C, vinblastine, vinorelbine, gemcitabine, cyclophosphamide, doxorubicin, epirubicin, methotrexate, 5-fluorouracil (5-FU), etc., have been evaluated (Bunn, 2002). Patients with metastatic breast cancer showed good clinical responses when treated with CDDP in combination with etoposide, a topoisomerase II inhibitor that inhibits cell-cycle
progression before mitosis (Decatris et al., 2004). Combination of CDDP with thymoquinone (TQ) has shown to help overcome CDDP resistance from over expression of NF-κB in lung cancer (Jafri et al., 2010). CDDP combination with cordycepin showed better apoptotic effect by activating caspase activation with possible MAPK pathway involvement in HNSCC cells (Chen et al., 2013). An effective and tolerable chemotherapeutic treatment of advanced or metastatic gastroenteropancreatic neuroendocrine carcinomas (GEP-NEC) is achieved when patients are given a combination treatment of irinotecan with CDDP (Lu et al., 2013).

Research in the field of platinum-based cancer chemotherapy showed that CDDP and analogous compounds exhibit very similar patterns of antitumor efficacy and susceptibility to resistance, thus the determining factors of cytotoxicity do not always follow the original structure-activity relationships. Hence, some studies suggest the possibly new clinical useful platinum-based anticancer agents to have novel structures unrelated to those agents assigned to platinum complexes (Abu-Surrah and Kettunen, 2006; Monneret, 2011; Marques, 2013) and also the extensive research developed in the area of drug design to find novel non-platinum-containing metal species with superior anticancer activity and low side effects (Chen et al., 2009). In recent years, several platinum compounds with trans geometry having distinctively different DNA binding modes from that of CDDP have proven to have higher in vivo and in vitro activity against CDDP-resistant cancer cells (Perez et al., 2000; Radulovic et al., 2002; Coluccia and Natile, 2007). Since the discovery of the activity of CDDP, researchers have tried to improve the drug by synthesizing and studying thousands of related compounds for their anticancer activity, which are referred to as analogs or second-generation drugs. After the efficacy and toxicity profiles of second-generation analogs are obtained, more analogs can be synthesized to form the third-generation drugs. Efforts to develop CDDP analogues having reduced toxicity and a broader range of therapeutic activity recently
led to release a few complexes to enter clinical trials (Galenski et al., 2005; Boulikas et al., 2007) of which carboplatin is accepted worldwide and oxaliplatin in a few countries (Wheate et al., 2010). Carboplatin, the most successful of second-generation platinum complexes, are CDDP derivatives that show less renal and gastrointestinal toxicity (Lokich and Anderson, 1998) as well as reducing nephrotoxicity (Wolfgang et al., 1994; English et al., 1999) as compared to CDDP toxicity. It has gained worldwide approval and steadily increasing acceptance as a less toxic alternative to CDDP. All “second-generation” drugs are administrated by intravenous injection or infusion. Though orally available drugs would give higher flexibility in the dosage and increase the potential use of platinum drugs, but for CDDP oral administration is not possible, due to its low levels of absorption. Recently, the so-called third generation platinum drug oxaliplatin also known as eloxatine has shown to have a broad spectrum of anticancer activity (Kim et al., 2010), and preclinical studies showed that oxaliplatin in combination with 5-FU has greater in vitro and in vivo anti-proliferative activity than either compound alone in several tumor models, including metastatic colorectal carcinoma (Zhang et al., 2006b; Saunders and Iveson, 2006). Oxaliplatin produces the same type of inter- and 1,2- GG intrastrand crosslinks as CDDP but has a spectrum of different activity and mechanisms of action and a lack of cross-resistance with CDDP and carboplatin (Di Francesco et al., 2002). Recently, some approaches have focused on applying Pt(IV) complexes such as satraplatin to have emerged as a novel oral platinum analogue with a better toxicity profile than CDDP, offering advantages in terms of patient convenience. Satraplatin and its major metabolite, JM118, have shown antineoplastic activity in vitro and in vivo, showing activity in advanced hormone-refractory prostate cancer (Sternberg et al., 2005; Armstrong and George, 2007). Use of satraplatin as an alternative platinum cytotoxic agent is particularly favoured because of the convenience of administration, milder toxicity profile, lack of cross-resistance with CDDP which is thought to be due
to a different detoxification mechanism, and activity in cancers historically non-responsive to platinum drugs (Choy et al., 2008; Bhargava and Vaishampayan, 2009).

In addition, new treatment techniques have also been applied— for example, the photoactivation (photoreduction) of some Pt(IV) complexes to the cytotoxic Pt(II) species by local application of UV or visible light to the tumor (Mackay et al., 2006; Butler et al., 2012) or electrochemotherapy treatment which consist of chemotherapy with platinum(II) drug followed by local application of electric pulses to the tumor to increase the drug delivery into cells (Marty et al., 2006; Sersa et al., 2009). The advantages of these techniques are their simplicity, short duration of the treatment sessions, low drug doses, and insignificant side effects (Sersa et al., 2008).

Cancer treatment by anticancer drugs reduces inherent antioxidants and induces oxidative stress which increases with disease progression. Although CDDP is one of the most widely used chemotherapeutic drug, its clinical use has been limited by its severe side effects, especially nephrotoxicity (Taguchi and Razzaque, 2005). In an attempt to overcome these impediments, the uses of CDDP in combination with some modulating agents have been tried with varying success (Treskes and Van der Vijgh, 1993; Zhang et al., 2001; Sanchez-Gonzalez., 2011). In an endeavour to decrease drug-induced toxicity in the host, the use of anticancer drugs such as cyclophosphamide (Nicol and Prasad, 2006; Prasad et al., 2010), paclitaxel (Park et al., 2012), arsenic trioxide (Yedjou et al., 2009), mitomycin C (Mazumdar et al., 2011), adriamycin (Devi and Latha, 2011) and ifosfamide (Donya et al., 2010) in combination with ascorbic acid (AA) have also been examined.

In the biological system, free radicals are often derived from oxygen, nitrogen and sulfur molecules. For example, ROS includes free radicals such as superoxide anion, perhydroxyl radical, hydroxyl radical, nitric oxide, hydrogen peroxide, etc. ROS are produced during cellular metabolism and functional activities, and have important...
roles in cell signalling, apoptosis, gene expression and ion transportation. However, excessive amounts of ROS can have deleterious effects on many molecules including protein, lipid, RNA and DNA since they are very small and highly reactive. ROS attacking macromolecules is often termed oxidative stress. In order to prevent or reduce the ROS-induced oxidative damage the human body and other organisms have developed antioxidant defense system which includes the enzymatic, metal-chelating, and free radical-scavenging activities to neutralize these radicals after they have formed.

In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body. Antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized. Many antioxidants may directly react with ROS and/or free radical intermediates induced by ROS and terminate the chain reaction, thereby stopping the ROS-induced damage.

Another important function of antioxidants is to regulate ROS-related enzymes. Antioxidants may decrease the cellular level of free radicals either by inhibiting the activities or expressions of free radical generating enzymes such as NAD(P)H oxidase (NOX) and xanthine oxidase (XO) or by enhancing the activities and expressions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

AA or vitamin C has been reported to be effective as a protectant against a variety of toxic chemical agents including heavy metals (Fox, 1975; Holloway and Peterson, 1984). The protective role of AA on CDDP-induced nephrotoxicity (Antunes et al., 2000a; Ajith et al., 2007) and mutagenicity (Giri et al., 1998; Antunes et al., 2000b; Nefic, 2001) have also been reported in both humans and animal model. Although the precise mechanism for the CDDP-induced toxicity is not well understood, it is preferentially taken up and accumulated in the liver and kidney cells (Stewart et al.,
1982), resulting in the enhanced production of ROS (Ajith et al., 2002; Mora et al., 2003; Kawai and Gemba, 2007; Itoh et al., 2011) and the decrease in antioxidant enzymes (Husain et al., 1996; Somani et al., 2000; Khynriam and Prasad, 2002). AA has been reported to increase the antineoplastic activity of CDDP, doxorubicin and paclitaxel against human breast carcinoma cells (Kurbacher et al., 1996). Therefore, antioxidants have been used with CDDP in the treatment to protect against CDDP toxicity (Somani et al., 2000; Ajith et al., 2007; Kilic et al., 2013).

1.4 ASCORBIC ACID (VITAMIN C) AND ITS CHEMOPREVENTIVE ROLE

Ascorbic acid (L, 3-ketothreohexuronic acid lactone, AA) commonly referred to as vitamin C is a water soluble antioxidant and an active reducing agent involved in various biological effects and plays an important role in the metabolism and detoxification of many endogenous and exogenous compounds (Henson et al., 1991). It is hydrophilic and an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage and generally functions as an antioxidant by directly reacting with reactive oxygen intermediates and has a vital role in defense mechanism against oxidative stress. It is synthesized in the liver in some mammals and in the kidney in birds and reptiles. However, several species-including humans, non-human primates, guinea pigs, Indian fruit bats, and Nepalese red-vented bulbuls are unable to synthesize vitamin C, and when there is insufficient vitamin C in the diet, humans suffer from the potentially lethal deficiency disease scurvy. Humans and primates lack the terminal enzyme in the biosynthetic pathway of AA, l-gulonolactone oxidase, because the gene encoding for the enzyme has undergone substantial mutation so that no protein is produced (Nishikimi et al., 1994).

In 1928, Szent-Gyorgyi, a Hungarian biochemist and Nobel Prize winner for studies on the biological function of AA, isolated a substance from adrenal glands
which he called ‘hexuronic acid’. Four years later, Charles Glen King isolated vitamin C in his laboratory and concluded that it was the same as ‘hexuronic acid’. Later, Norman Haworth deduced the chemical structure of vitamin C in 1933 for which he was awarded the Nobel Prize for chemistry in 1937. McCormick in 1954 postulated that ascorbate protects against cancer by increasing collagen synthesis. In 1972, Cameron and Rotman hypothesized that ascorbate could have anticancer action by inhibiting hyaluronidase and thereby preventing cancer spread. These hypotheses were subsequently popularized by Cameron and Pauling who reported in 1978 that high dose vitamin C (typically 10g/day, by intravenous infusion for about 10 days and orally thereafter) increased the average survival of advanced cancer patients up to 20 times longer than that of controls.

AA molecule is a six-carbon compound with four hydroxyl groups in positions 2, 3, 5 and 6 (Figure 4). The molecular formula of AA is C₆H₈O₆. It has a molecular mass of 176.12 and melting point about 190°C (with decomposition). It is structurally related to glucose and consists of two inter-convertible compounds: L- ascorbic acid, which is a strong reducing agent, and its oxidized derivative, L-dehydroascorbic acid. It is readily oxidized, especially in aqueous solutions, by reacting with atmospheric oxygen, and act as an electron donor to free radicals such as hydroxyl and super oxide radicals. It is very sensitive to even slight heating and to the action of oxidizing agents and metal ions.

AA has a long history of adjunctive use in cancer therapy but the definite use of vitamin C for the treatment of cancer still remains inconclusive (Verrax and Buc Calderon, 2008). While many studies have reported good therapeutic potential of AA against cancer (Kathleen, 1998; Ohno et al., 2009), some have shown virtually no benefit from AA treatment (Creagan et al., 1979; Moertel et al., 1985).
In general, AA can potentially help cancer patients by its role in collagen production which may protect normal tissues from tumor invasiveness and metastasis, while in cancer patients, replenishment of AA may improve immune system function and enhance patient’s health. The role of AA in the maintenance of tissue integrity (Wilson, 1977) and host defense (Field et al., 2002) provides a rational basis for examining their relationship to cancer. The antioxidant and scavenging properties of AA holds promising prospect in cancer prevention and treatment. Antioxidants represent a first line body defense against oxidative stress produced by generation of free radicals and ROS. The chemotherapeutic/therapeutic role of AA against cancers has been widely reported (Cameron et al., 1979). Ghosh and Das (1985), though the actual involvement of AA in the development and progression of tumors is not clearly understood (Chen et al., 1988).

AA is also involved in collagen biosynthesis, cytochrome P-450 dependent hydroxylase activities, maintenance of polysomes, stimulation of chemotaxis, phagocytosis, protection against infection, detoxification processes, stimulation of the immune system, wound healing, prevention of oxidation, etc. As far as the mechanism of the observed effect of AA is concerned, individual researchers proposed various possible mechanisms of AA activity in the prevention and treatment of cancer which includes: enhancement of the immune system; stimulation of collagen formation.
necessary for “walling off” tumors; inhibition of hyaluronidase which keeps the ground substance around the tumor intact and prevents metastasis; prevention of oncogenic viruses; correction of an ascorbate deficiency often seen in cancer patient; expedition of wound healing after cancer surgery; enhancement of the effect of certain chemotherapy drugs such as CDDP, cyclophosphamide, etc.; reduction of the toxicity of other chemotherapeutic agents such as CDDP, adriamycin, etc.; prevention of free radical damage; and neutralization of carcinogenic substances.

Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical). The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalysts.

1.4.1 Antioxidant action of AA

Under physiological conditions, vitamin-C predominantly exists in its reduced form, ascorbic acid (AA) or ascorbate; it also exists in trace quantities in the oxidized form, DHA (dehydroascorbic acid). At physiological pH, ascorbate occurs in the monoanion form. Loss of the first electron results in the formation of ascorbate free radical (AFR) which is not very reactive, and mild oxidants such as Fe$^{3+}$($\text{CN}$)$_6$ (ferricyanide) remove a second electron and convert the AFR to DHA and itself gets reduced to Fe$^{2+}$($\text{CN}$)$_6$ (ferrocyanide) (Figure 5). The plasma membrane oxidoreductases (PMOR), a multienzyme complex that includes NADPH-ferricyanide reductase and NADPH oxidase along with cytochrome-B5 reductases play a vital role in the conversion of AFR to DHA. There are two known mechanisms for transporting AA into the cells. A universal system, present in all cells, transports intracellular levels AA as DHA via facilitative glucose transporters (GLUTs). Once inside the cell, DHA is rapidly reduced and accumulates as AA, either directly by the action of GSH or in
reactions catalyzed by thioredoxin reductase (TxnRd) or glutaredoxin (Glrx). NADH-dependent mechanisms may also contribute to this mechanism. TxnRd and Glrx, also fall under this category of ROS-sensitive signal transducers. The second transport system is functional in cells where AA is directly transported into cells via sodium-dependent ascorbate co-transporters or sodium-dependent vitamin C transporters (SVCTs) (Wilson, 2005). The human granulocyte-macrophage colony-stimulating factor (GMCSF) phosphorylates granulocytes-macrophage colony-stimulating factor receptors (CSFRs) and facilitates janus kinase-2 (JAK2) activation for an enhanced uptake of DHA through GLUTs. This results in increased intracellular levels of AA. GLUTs do not transport ascorbate in vivo. Increased levels of AA and DHA suppress the formation of ROS and activation of Ikappa B kinases (IKKs) to induce the antioxidant defense and cooperation (and/or compensation) between different antioxidant systems are the determinants of the competence of the antioxidant system (Carcamo et al., 2004). Such mechanisms substantiate the importance of ROS in cytokine and growth factor signaling and indicate a role for AA in down modulating such signaling responses to counteract stress conditions within tissues. This action point to AA as a potent antioxidant and a regulator of redox-signal transduction in host defense cells and therefore has a possible role in controlling inflammatory responses (Guaquil et al., 2004).

AA, being an important water-soluble biological antioxidant, scavenging ROS, its chemopreventive effects in carcinogenesis may be linked to the protective effects of AA against epigenetic mechanisms such as inflammation and inhibition along with its ability to detoxify carcinogens such as 2-aminoanthracene, benzo(a)pyrene, 2-nitrofluorene, and nitrofen (Cabrera, 2000) as well as its ability to block carcinogen processes through antioxidant activities.
Although AA has been known to stimulate immune function and block the metabolic activation of carcinogens, its cancer-preventive effects may be associated mainly with its protective effects against oxidative stress. The generation of oxidative...
stress is an integral part of the inflammatory response associated with tumor promotion. AA was shown to reduce inflammation caused by reactive oxygen intermediates thereby attenuating gastric cancer (Feiz and Mobarhan, 2002). Carr and Frei (1999) showed that AA predominantly reduces oxidative damage \textit{in vivo} even in the presence of iron. The administration of the antioxidant AA enhances the contractile response to dobutamine and improves myocardial efficiency in patients with heart failure (Shinke et al., 2007).

Besides the antioxidant activity, an intrinsic pro-oxidant potential of AA may contribute to its chemopreventive properties. One study suggests that the pro-oxidant form of AA may upregulate some of the enzymes involved in DNA repair. Inhibition of cell-to-cell communication strongly promotes tumor formation. Role of AA has also been suggested in inhibiting carcinogenesis (Dunham et al., 1982; Lee et al., 2002; Surjyo and Anisur, 2004) or enhancing carcinogenesis (Banic, 1981; Fukushima et al., 1983). In an \textit{in vitro} study, AA has shown to inhibit the mutagenic actions of carcinogenic \textit{N}-nitroso compounds (Guttenplan, 1978). Some genotoxic effects of vitamin C \textit{in vitro} test systems has also been demonstrated (Shamberger, 1984; Nefic, 2008) but in the experiments \textit{in vivo}, there are no genotoxic effects by vitamin C (Apsel et al., 2011). AA also exerts protective effects against the disruption/inhibition of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) induced gap-junctional intercellular communication (GJIC) (Lee et al., 2002). Several reports have revealed the growth inhibitory effect of AA on tumor cells in experimental animals. Combination of AA and vitamin K\textsubscript{3} exerts its antitumor and antimetastatic activities by inhibiting the metastases of mouse liver tumors in C3H mice both \textit{in vitro} and \textit{in vivo} (Taper et al., 2004). Pharmacological AA inhibits both the growth and the metastatic potential of hormone refractory PAIII cells when implanted in a syngeneic, immune-competent rodent system (Pollard et al., 2010). It has been reported that AA status of the host is often low in malignant conditions (Krasner and Dymock, 1974; Moncayo et al., 2008). AA and its derivatives were shown
to be cytotoxic and inhibited the growth of a number of malignant and non-malignant cell lines *in vitro* and *in vivo* (Park and Kimler, 1991; Roomi et al., 1998). In contrast to these observations, results of some studies indicate that AA may increase or accelerate tumor growth (Barett, 2008). Although AA at high doses inhibited tumor growth in mice (Yeom et al., 2009), but low doses accelerated tumor growth. It is reported that administration of AA potentiated the growth of transplanted solid tumors in mice (Liotti et al., 1983).

AA has been known to be utilized by the animals for maintenance of their defense mechanism that include immune competence and phagocytosis. AA at a nontoxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition of tumor cells in culture *in vitro* and *in vivo* condition (Sarna and Bhola, 1993; Roomi et al., 2011; Vuyyuri et al., 2013). The sequence-dependent synergistic antitumor activity between AA and CDDP against murine ascites Dalton’s lymphoma (DL) was reported by Prasad et al. (1992), leading to tumor regression with significant increase in hosts survivability. Synergistic combination of vitamin K₃ and AA induces cell death in different cancers (De Loecker et al., 1993; Tareen et al., 2008; Tomasetti et al., 2010). The influence of AA and 6-chloro-6-deoxy ascorbic acid (6-Cl-AA) serves as potential antitumor agents, on the growth of various human cell lines especially those that are resistant to chemotherapy (Osmak et al., 1997). AA supplementation resulted in decreased tumor growth and enhanced encapsulation of tumors and inflammatory cytokine secretion (Cha et al., 2011).

The effect of AA against different cancers is variable depending on the carcinogen-induced cancer, doses, and route of administration and different species of animals (Migliozzi, 1977). AA at concentrations as low as 10μM and 50μM have been reported to diminish the DNA damage evoked by the CDDP selenium conjugate
Combination of AA and \( \text{H}_2\text{O}_2 \) at non-toxic doses, significantly decrease the GSH levels in the cancer cells leading to effective death of cancer cells (Hardaway et al., 2011). The ability of AA to confer marked protection to the animals against many toxic chemical agents and heavy metals has been described (Ali et al., 2011; El Shafai et al., 2011). Dietary AA protects human sperm from endogenous oxidative DNA damage that could affect sperm quality and increase risk of genetic defects, particularly in smokers having low AA (Fraga et al., 1991). The protective role of AA in 2,4- dichlorophenol induced teratogenic/ carcinogenic activity along with significantly increased liver AA and GSH levels at high dose has also been reported (Nagyova and Ginter, 1994). Recent pharmacokinetic data also suggests that pharmacologic concentrations of AA can be achieved by intravenous administrations which may serve as a safe, adjunctive therapy in clinical cancer care (Chen et al., 2005; Mikirova et al., 2013).

Thus, considering the variable findings on the significance of AA in relation to cancer chemotherapy and the possibility of development of CDDP induced side effects, the present study is aimed to investigate on the various cellular and biochemical/ultrastructural parameters in tumor cells as well as other tissues in an attempt to understand the antitumor activity and biochemical changes in the tumor-bearing hosts treated with CDDP/AA alone or in combination with AA plus CDDP and also to determine the toxicity in the hosts treated with CDDP alone or in combination with AA.