Literature Review
Cancer is a class of disease in which a cell, or a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology. Cancer can affect people of all ages with the risk for most types increasing with age (Cancer Research UK, 2007). It caused about 13% of all human deaths in 2010 (World Health Organization, 2010). Cancer is primarily an environmental disease with 90-95% of cases due to lifestyle or environmental factors and 5-10% due to genetics (Anand et al., 2008). Common environmental factors leading to cancer death include: tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), environmental pollutants. These environmental factors cause abnormalities in the genetic material of cells (Kenneth, 2004).

Medical science has developed number of ways for treatment of cancer which include chemotherapy, radiation therapy, adjuvant therapy and the newer targeted therapies, as well as are refining surgical techniques for removing cancer. Chemotherapeutic drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells. As these drugs cause damage to cells they are termed cytotoxic. There are a number of strategies in the administration of chemotherapeutic drugs used today. Chemotherapy may be given with a curative intent or it may aim to prolong life or to palliate symptoms (Skeel, 2003). Combined modality chemotherapy is the use of drugs with other cancer treatments, such as radiation therapy or surgery. Most cancers are now treated in this way. Combination chemotherapy is a similar practice that involves treating a patient with a number of different drugs simultaneously. The drugs differ in their mechanism and side effects. The biggest advantage is minimizing the chances of resistance developing to any one agent. The majority of chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. Antimetabolites which include Methotrexate and 5-Fluorouracil masquerade as purines (azathiopurine, mercaptopurine) or pyrimidines which become the building blocks of DNA. They
prevent these substances from becoming incorporated in to DNA during the "S" phase of the cell cycle, stopping normal development and division (Chabner and Longo, 2005; Camphausen and Hoskins, 2008).

**Methotrexate**

Methotrexate (MTX) is an anticancer drug which is used to treat choriocarcinoma, leukemia in the spinal fluid, osteosarcoma, breast cancer, lung cancer, non-Hodgkin lymphoma, and head and neck cancers. Several earlier studies have revealed that MTX is taken up by the cells and tissue which is then immediately converted to metabolites linked to glutamate (MTX-polyglutamate). The resulting complex is responsible for most of biochemical and biological activities of MTX (Panetta et al., 2002). MTX-polyglutamate inhibited enzymes such as dihydrofolatereductase (DHFR) and several other that are dependent on folate. This drug appears to block biosynthesis of nucleotides (Budzik et al., 2000) by inhibiting DHFR, resulting in the disruption of DNA biosynthesis (fig. 1); this is the basis of the chemotherapeutic action of MTX as well as other DHFR inhibitors, generically known as antifolates (Navarro-Perán et al., 2005).

Another suggested mechanism of MTX immunosuppressive action is by mediating the release of adenosine, thus suppressing inflammation (Cronstein et al., 1993; Morabito et al., 1998). The release of some interleukins and eicosanoids is known to be decreased by MTX, but the mechanism is still unclear (Rudwaleit et al., 2000; Schuerwegh et al., 2001). Recently, the antiinflammatory action of MTX has been related to the induction of apoptosis. MTX was found to induce apoptosis in activated healthy T cells (Fairbanks et al., 1999; Genestier et al., 1998; Nishioka et al., 1998; Antosiewicz et al., 2006). A connection to apoptosis was suggested for Jurkat T cells and U937 monocytes (Strauss et al., 2002; Xiao et al., 2002). In addition, the apoptotic effect of MTX was also found in CD4+ cells (Antosiewicz et al., 2006).
Fig. 1. Mechanism of Action – competes with folic acid for the active binding site dihydrofolate reductase (DHFR) enzyme (Budzik et al., 2000)
MTX belongs to a pterine group of compounds and is also used as a photosensitizer which photo-dissociates into 6-formylpterin and p-aminobenzooylglutamic acid upon UV-Vis light exposure (Brezeanua et al., 2004) fig. 2. The Kazutaka (2002) has shown through molecular orbital study that aminobenzoyl moieties of MTX are the electron-donating groups, suggesting that the fluorescence from their pteridine moieties can be quenched through intra-molecular electron transfer from their aminobenzoyl moieties. It has also been reported that the C9-N10 bonds connecting the pteridine and aminobenzoyl moieties of MTX are unstable against oxidative agents. Singlet oxygen mediated cleavage of this C9-N10 bond or oxidation of the electron-donating moiety should recover the fluorescence intensity of their pteridine moieties. In general, pteridine compounds absorb the ultraviolet A region and show fluorescence in the visible-light region. MTX scarcely absorb visible light, indicating that visible light irradiation itself does not decompose these molecules. Photosensitized MTX in the presence of methylene blue in distilled water shows strong fluorescence in the visible light region, although the fluorescence of MTX itself could not be detected. This fluorescence can be assigned to the π*–κ transition of the pteridine compound (Fairbanks et al., 1999).

The mechanism of damage to biomacromolecules induced by photoirradiated MTX was also examined by Kazutaka et al., (2002) using 32P-labeled DNA fragments obtained from a human gene. Photoirradiated MTX caused DNA cleavage specifically at the underlined G in 5’-GG and 5’-GGG sequences in doublestranded DNA only when the DNA fragments were treated with piperidine, which suggests that DNA cleavage was caused by base modification with little or no strand breakage. With denatured single-stranded DNA the damage occurred at most guanine residues. The amount of formation of 8-hydroxy-2’-deoxyguanosine (8-oxodGuo), an oxidative product of 2’-deoxyguanosine, in double-stranded DNA exceeded as compared to single-stranded DNA. These results suggest that photoirradiated MTX participates in 8-oxodGuo formation at the underlined G in 5’-GG and 5’-GGG sequences in double-stranded DNA through electron transfer, and then 8-oxodGuo undergoes further oxidation into piperidine-labile products. Kazutaka et al., (2002) has also shown through fluorescence measurement, high-pressure liquid chromatography and mass spectrometry that photoexcited MTX is hydrolyzed into 2,4-diamino-6-(hydroxymethyl) pteridine (DHP). DNA damage induced by DHP was observed in a
similar manner as was the damage induced by MTX. The extent of DNA damage and the formation of 8-oxodGuo by DHP were greater than those induced by MTX. The kinetic analysis, based on the time course of DNA oxidation by photoirradiated MTX, suggests that DNA damage is resulting in the formation of DHP, which exhibits a phototoxic effect caused by oxidation of biomacromolecules through photoinduced electron transfer.
Literature Review

Fig. 2. Methotrexate upon photoillumination (Brezeanua et al., 2004)

Methotrexate

\[ \text{Illumination} \]

2,4-diamino-6-(hydroxymethyl)pteridine  p-(N-methylamino)benzoyl-L-glutamic acid
Methotrexate and Photodynamic Therapy

The efficacy and cancer selectivity of ALA-based PDT can be enhanced by using methotrexate as a preconditioning agent. Methotrexate provides a significant tumoricidal activity on its own and also enhances the selective production of protoporphyrin IX (PpIX) within carcinoma cells when used at low concentration. PpIX then serves as the target for light. Although the skin carcinoma cell lines intrinsically produced ~3-fold higher levels of PpIX than did normal epithelial keratinocytes, this difference did not appear to offer any selective advantage for photodynamic killing in the absence of methotrexate. However, when cells were preconditioned with methotrexate, a significant induction of PpIX levels occurred only in the cancer cell lines, and this induction translated into selective and efficient photodynamic killing of the carcinoma cells. Cytotoxic enhancement due to the combination of methotrexate and ALA-PDT was reported more than additive (synergistic) in the monolayer cultures. Importantly, very low methotrexate concentrations (~2 nmol/L) proved to be quite effective at enhancing ALA-PDT (Jaroslav et al., 2002). In vivo study conducted by Sanjay et al., (2009) has also explored that PpIX levels are enhanced at very low, nontoxic concentrations of methotrexate. Stating, methotrexate was shown to enhance PpIX levels ~2-fold in a differentiating (organotypic) epidermal model. Second, PpIX enhancement of ~6-fold was shown in skin tumors produced by chemical carcinogenesis in mice. Third, PpIX enhancement of ~3-fold was shown in subcutaneous human A431 tumors in nude mice. This has important clinical implications. Oral methotrexate and ALA-PDT for skin carcinoma could lead to a safe and effective new treatment regimen. Thus, PpIX levels can be induced in carcinoma cells using low, nontoxic doses of methotrexate. Inducible changes in mitochondrial CPO expression and subsequent accumulation of PpIX appear to underlie this response. The cytotoxic (therapeutic) effect achieved by combining methotrexate and ALA-PDT is synergistic in cell culture and is also significant in organotypic and in vivo animal models.

Methotrexate and nanoparticles

To retain MTX within tumor cells for longer duration and alter its pharmacokinetic behavior several studies were conducted which showed the
formulation of MTX bound nanoparticle that served as drug carriers. In one study, the MTX–AuNP conjugate was developed and its cytotoxic effect as examined in vitro and antitumor effect in vivo. Spectroscopic examinations revealed that MTX can be directly bound onto AuNP via the carboxyl group (–COOH) to form the MTX–AuNP complex which is kinetically released from the nanoparticles. The accumulation of MTX is faster and higher in tumor cells treated with MTX–AuNP than that treated with free MTX. Notably, MTX–AuNP shows higher cytotoxic effects on several tumor cell lines compared to an equal dose of free MTX. This can be attributed to the “concentrated effect” of MTX–AuNP. Administration of MTX–AuNP suppresses tumor growth in a mouse ascites model of Lewis lung carcinoma (LL2), whereas an equal dose of free MTX had no antitumor effect. In conclusion, these results suggest that by combining nanomaterials with anticancer drugs like MTX–AuNP may be more effective than free MTX for cancer treatment (Yu-Hung et al., 2007).

5-Fluorouracil

5-Fluorouracil (5-FU) is the class of compounds that has been subjected to intensive research as a chemotherapeutic agent. Colorectal cancer (CRC) is the second highest cause of cancer death in Western countries. About 50% of all patients with CRC develop distant metastatic disease and will be candidates for palliative chemotherapy (Seium et al., 2005). The prognosis of colorectal cancer is principally based on the stage of the disease at the time of detection. Therapy for advanced colorectal cancer can be divided into colon and rectal cancer components. Rectal cancers are treated surgically by low anterior resection or abdominal perineal resection, often in combination with 5-FU-based chemoradiation. It is important to note the systemic therapeutic progress made in improving survival of patients with metastatic colorectal cancer. Before the 1960s, patients with untreated metastatic colorectal cancer had a median survival of 4-6 months. With the introduction of 5-FU treatment, patient survival was extended to 11–12 months, and 5-FU continues to form the core for colorectal cancer therapy. In the 1980s, biomodulators such as leucovorin were added to 5-FU, improving the survival by an additional 2–4 months. Irinotecan and later oxaliplatin were added to 5-FU as second-line treatments, this was later approved as first-line therapies for metastatic colorectal cancer, further
improved overall survival to 15-17 months after diagnosis. A metaanalysis of 13 randomized control trials of 1365 patients treated with systemic chemotherapy for metastatic colorectal cancer shows a 35% (95% CI: 24–44%) reduction in the risk of death, translating into an improvement in median survival of 3.7 months. Carethers JM, (2008) conducted Pilot study; eighteen patients with locally advanced unresectable rectal carcinoma were treated with radiation therapy by 45 or 55 Gy combined with continuous chronobiologically modulated 5-FU infusion. Daily doses of 5-FU started at 250 mg/m², and escalated by 25 mg after a group of 5 patients experienced no grade 3 toxicity. This pilot study showed excellent results i.e., all the patients were able to undergo potentially curative surgery with 28% pathologic and 22% clinic complete response thus 5-FU induction along with a radiation exposure may enhances the treatment of rectal carcinoma (Sobat et al., 1999).

5-FU is a potent radiosensitizer in colon and rectal cancers, and acts in a similar manner as 5-bromouracil, its main biochemical action is as an antimetabolite of the uracil anabolic pathways. The cytotoxic effect of 5-FU in most systems is attributed primarily to its anabolism to 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthase, a pivotal enzyme in pyrimidine biosynthesis. Thymidylate synthase (TS) is a vital enzyme for the growth of tumors, and the expression of this gene is dependent on the cell cycle. 5-FU shows greater selectivity against solid tumors with multi-resistance to drugs than do other antitumor agents (Takeo et al., 2008). 5-FU is typically administered with the biomodulator leucovorin, a reduced form of folate that stabilizes the binding between 5-FU and thymidylate synthase (Carethers, 2008). The exact molecular mechanisms that mediate events downstream of TS inhibition have not been fully elucidated. Depletion of dTMP results in subsequent depletion of deoxythymidine triphosphate (dTTP), which induces perturbations in the levels of the other deoxynucleotides (dATP, dGTP and dCTP) through various feedback mechanisms. Deoxynucleotide pool imbalances (in particular, the dATP/dTTP ratio) are thought to severely disrupt DNA synthesis and repair, resulting in lethal DNA damage (Fig. 2). In addition, TS inhibition results in accumulation of dUMP, which might subsequently lead to increased levels of deoxyuridine triphosphate (dUTP) (Daniel et al., 2003).
5-Fluorouracil (5-FU) is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP respectively as shown in fig. 3.

An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver.
Fig. 3. Metabolism of 5-Fluorouracil (Sobat et al., 1999)
High intracellular levels of the reduced folate CH₂THF are necessary for optimal binding of FdUMP to TS. Leucovorin (LV, 5'-formyltetrahydrofolate) has been used to expand the intracellular concentration of CH₂THF and has been shown to increase the *in vitro* and *in vivo* toxicity of 5-FU in many cancer cell lines. LV enters the cell via the reduced folate carrier and is anabolized to CH₂THF, which is then polyglutamated by folylpolyglutamate synthetase. Polyglutamation not only increases the cellular retention of CH₂THF, but also enhances the stabilization of its ternary complex with TS and FdUMP (Daniel et al., 2003).

It was also shown that Pyrimidine bases undergo dimerization to form various modes of chemical linkages by photochemical, radiation and electrochemical reactions. There is considerable mechanistic evidence of the pyrimidine dimerizations involving successive transfers of an electron and a proton: (1) one-electron reduction of the pyrimidine followed by protonation to generate a carbon-centered free radical which results in a dihydrothymine dimer with a single C-C linkage; (2) one-electron oxidation of the pyrimidine followed by deprotonation to generate a carbon-centered or nitrogen-centered free radical which results in pyrimidine dimer with a single C-C or N-C linkage. Pyrimidine radical cations as produced by the one-electron oxidation have been a subject of extensive studies by pulse radiolysis and laser photolysis, because they are intermediate species of biological importance that would result from the direct action of ionizing radiation to cause oxidative damage of DNA (Hatta et al., 2001).

Galvanostatic electrolysis or radiation-induced oxidation of aqueous 5-FU solution yields the N1–C5-linked dimer hydrate of 5-FU via coupling between the N1 centered radical and C5-centered radical of 5-FU (Takeo et al., 2008). Interferons (IFNs) are pleiotropic cytokines that exert negative regulatory effects on the growth of normal and malignant cells *in vitro* and *in vivo*. Numerous *in vitro* studies have reported that 5-FU interacts with IFNs to produce greater than additive cytotoxicity in various cancer cell lines. Moreover, it was found that IFN-α enhanced 5-FU-mediated single- and double-strand DNA breaks in colon carcinoma cells. IFN-γ has been reported to upregulate the activities of the 5-FU anabolic enzymes TP and UP, resulting in enhanced 5-FU activation. A study by Daniel et al., (2003) found that
acutely translational upregulation of TS expression by 5-FU (see below) was abrogated by IFN-γ in the H630 colon cancer cell line, resulting in enhanced TS inhibition.

The tumour suppressor p53 maintains DNA integrity by transcriptionally activating genes such as CDKN1A and GADD45α, the products of which induce cell-cycle arrest in response to DNA damage. However, depending on the cellular context and the nature of the DNA damage, p53 can trigger elimination of the damaged cells by promoting apoptosis through the induction of pro-apoptotic genes, such as FAS (CD95/APO1) and bax, and the downregulation of anti-apoptotic bcl-2 (REFS). In vitro studies have reported that loss of p53 function reduces cellular sensitivity to 5-FU. It has been shown that disrupting both alleles of TP or bax in a colon cancer cell line made the cells strikingly resistant to apoptosis induced by 5-FU compared with the parental line. Moreover, it was also shown that p53 stabilization in response to TS inhibition by the TS-targeted antifolate raltitrexed was abrogated in breast cancer cells by increased TS expression, whereas 5-FU-mediated induction of p53 was insensitive to increased TS expression (Daniel et al., 2003).

Copper

All transition metals, with the exception of copper and Iron contain one electron in their outermost shell and can be considered free radicals. Copper has a full outer shell, but can lose or gain electrons very easily making itself a free radical. This property makes copper common catalyst for oxidation reactions (Halliwell and Gutteridge, 1985). Copper is a redox-active metal that is predominantly used by organisms living in oxygen-rich environments and that fluctuates between the oxidized (Cu²⁺) and reduced (Cu⁺) states. With these changes in redox state, copper can coordinate to a range of ligands that include carboxylate oxygen, imidazole nitrogen, cysteine thiolate, and methionine thioether groups and engages in cation-Π interactions (Michelle et al., 2009).

Over the past few decades, critical progress has been made in the identification of genes encoding proteins that function in copper uptake, intracellular distribution, and efflux and in the regulation of the copper homeostasis machinery. Integral membrane protein, functions as a major copper importer at the plasma membrane. The genetic requirement for a metallo reductase in yeast for integral
membrane protein-mediated high affinity copper uptake, in addition to other data, supports the transport of Cu$^+$ rather than Cu$^{2+}$. Conserved methionine residues are present in the extracellular domain as Met-X-Met or Met-X2–Met. Additionally, a conserved Met motif, Met-X3–Met, present in the integral membrane protein which is essential for Cu$^+$ import (Michelle et al., 2009).

Copper, which is absorbed into the body through a membrane protein, is necessary to the healthy functioning of the human body. A deficiency can give rise to disease, while loss of regulation is toxic. Therefore, the cell handles copper ions with special care. One chaperone molecule delivers the copper ion to an "entrance gate" outside the cell; another chaperone then picks it up and carries it to various destinations inside the cell. The researchers suggest that this delicate system is maintained by passing one copper ion at a time by the copper transporter, allowing for maximum control of the copper ions. This way, there is no risk of bringing several copper ions into the protein at the same time, which ultimately prevents harmful chemical reactions between the ions and the abundant chemical reagents within the cell (Maya et al., 2010).

In the body free copper ions are not normally available but are bound tightly to serum albumin or incorporated into careuloplasmin. The tendency of copper ions to bind readily to amino groups of proteins has often made it appear that proteins will thus prevent copper-ion dependent *OH formation. Actually, the *OH generation is not prevented but rather localized to the site of binding of the copper ions, and the protein molecule itself will be damaged by the *OH radicals. Histidine residues have been particularly implicated as sites of copper-ion-dependent protein degradation. In fact, the effect of complexing catalytic metal ions on *OH production is not readily predictable. Chelating agents may promote metal-ion-dependent *OH formation (e.g. Fe3+-EDTA) or inhibit it (e.g. Fe3+-desferal), depending on a number of factors including (i) the solubility of the complex, (ii) the redox potential of the Mn$^{3+}$/Mn$^{2+}$ couple and consequently its ability to be reduced by O$_2$•− and (iii) whether or not it has a free co-ordination site and so is able to catalyse H$_2$O$_2$ breakdown (Jeremy et al., 1998).

The role of copper in the development of cancer is somewhat similar to its role in cardiovascular disease. This is because the serum level of copper is often elevated
in animals and humans with cancer (Oberley and Buettner, 1979). Like the elevation of serum copper in cardiovascular disease, it seems that the elevation of serum copper that occurs in conjunction with cancer is part of the body’s biological response to the cancer, rather than its cause. Numerous studies examining varied types of tumors have demonstrated that with remission usually comes a decrease in serum copper levels to normal. Patients who responded to therapy or surgery usually return to normal serum copper levels, while non responders had a persistently elevated serum copper level. Interestingly, most tumor cells have decreased Cu-Zn SOD activity compared to normal cells, and it has been suggested that the elevation in serum copper is a physiological response designed to activate SOD or other copper enzymes in cancer cells to inhibit their growth. Indeed, numerous copper complexes that demonstrate SOD-mimetic properties, including copper salicylate, have been shown to possess anticancer, anticarcinogenic, and antimutagenic effects both in vitro and in vivo (Sorenson, 1989).

**Effect of Pro-oxidants in presence of transition metals**

Pro-oxidants are chemicals that induce oxidative stress, either through inducing reactive oxygen species (ROS) or inhibiting antioxidant systems (Puglia and Powell, 1984). Some substances can act as either antioxidants, or pro-oxidants, depending on the specific set of conditions (Aleksandra et al., 2007). Some of the conditions that are important include the concentration of the chemical and the presence of oxygen or transition metals. The reduction of oxygen typically involves either the initial formation of singlet oxygen, or spin-orbit coupling through a reduction of a transition-series metal such as manganese, iron, or copper (Herbert, 1996).

Many antitumor drugs are cytotoxic due to their ability to generate free radicals, characterizing specific metabolic pathways that may regulate the pro-oxidant action of antitumor drugs therefore, will be essential for optimizing clinical efficacy (Omaye et al., 1997). Anticancer drugs can exert prooxidant effects by auto-oxidation, in which the initial step leads to superoxide (O$_2^-$) formation. Superoxide anion is very reactive and may damage major macromolecules such as proteins or DNA. Moreover, superoxide anion and superoxide radical will accelerate auto-oxidation, generating
H$_2$O$_2$ in the process (Omaye et al., 1997). Thus, increase in prooxidant activity of anticancer drugs in the presence of Fe (III) or Cu (II) was primarily associated with their ability to reduce metal ion. Subsequently, Fe (III) and Cu (II) can be reoxidized in Fenton-type reactions leading to the production of ‘OH and other ROS (Aleksandra et al., 2007).

**Copper-dependent Metabolic Changes in Cancer Cells**

Recent exciting work has implicated copper-handling and copper-utilizing proteins in controlling the striking metabolic changes that have long been known to occur in cancer cells. Otto Warburg, (1956) discovered that respiratory capacity is down-regulated in many cancer cell types with a concomitant dependence on glycolysis for cellular energy generation. Although this phenomenon has been known for over 75 years, the cellular regulatory mechanisms governing this metabolic switch are still not well understood. Michelle et al., (2009) reasoned that this metabolic re-engineering may result from regulatory changes common to many cancer cell types. Because mutations in the gene encoding the p53 sequence-specific DNA-binding transcription factor are among the most common genetic changes found in a broad range of cancer cells, they explored whether cancer cell lines harboring p53 mutations exhibit changes in their dependence on respiration versus glycolysis. Indeed, human colon cancer cells with p53-inactivating mutations also showed significant reductions in oxygen consumption while generating increased levels of lactate, a by-product of glycolysis, metabolic characteristics reflecting a switch from oxidative phosphorylation to glycolysis is typical of the Warburg effect.

**Copper in Signaling and Tumor Cell Metastasis**

Cell migration and tissue invasion from the primary tumor site require a loss of integrity of target tissue cell-cell junctions. The epithelial-mesenchymal transition is a critical step in the initiation of cell invasion, as it results in compromised cell-cell junctions due, at least in part, to the down-regulation of E-cadherin, a protein that functions in cell-cell adhesion. Normally, the transcription of E-cadherin is regulated by the Snail repressor, the protein stability of which is tightly regulated through its phosphorylation by glycogen synthase kinase 3β. Recent work has identified lox-like proteins to also play a part in the regulation of Snail protein stability. Although the
extracellular copper-dependent lox is well established for its role in the maturation of collagen and elastin via lysine oxidation, four additional members of this protein family, called lysyl oxidase-like proteins (LOXL), have been identified. All five members of these family harbor signatures for bound copper and lysyl-tyrosyl quinone cofactors, but LOXL lack a typical secretory propeptide found in extracellular lox and instead contain scavenger receptor cysteine-rich domains and are predicted to be intracellular proteins. Recent studies have demonstrated that LOXL2 and LOXL3 interact with Snail to stabilize the protein in a manner that is dependent on two Snail lysine residues. These and other studies support a model to suggest that lysine oxidation diminishes glycogen synthase kinase 3β-mediated phosphorylation, thereby stabilizing Snail, inhibiting E-cadherin expression, and compromising tight junctions. Interestingly, LOXL2 expression is strongly elevated in highly invasive forms of metastatic breast, colon, and esophageal cancer, consistent with a more general role in cancer cell invasion (Michelle et al., 2009).

**Copper and Cancer Chemotherapy**

Copper has been proposed to be essential for angiogenesis, the formation of new blood vessels that provide a delivery route for nutrients, growth factors, and other signaling agents important for tumor growth and survival. Although a number of copper dependent roles in angiogenesis have been proposed, the entire range of functions important for efficient angiogenesis that require copper has not been elucidated. This is an important challenge, as Tetrathiomolybdate (TTM), a potent Cu\(^{+}\) chelator, has been reported to be of therapeutic value in the treatment of several types of cancers as an anti-angiogenesis and anti-cancer molecule. Recently, ATN-224, an orally available choline salt derivative of TTM, has been suggested to preferentially target Cu, Zn-SOD in tumor and endothelial cells, with the implication that SOD1 plays a stimulatory role in growth factor signaling. SOD1 protects cells from oxidative stress and catalyzes the conversion of superoxide to hydrogen peroxide, molecules that have been established to play signaling roles in biology. In an initial report, TTM was shown to potently inhibit Cu, Zn-SOD activity, thereby increasing superoxide anion accumulation and inducing programmed cell death in multiple myeloma cells. Parallel with these features, the phosphorylation of extracellular signal-regulated kinase (ERK), which has critical functions down stream
of Ras in cell proliferation, was also inhibited. Recently, ATN-224 was reported to inhibit growth factor-stimulated phosphorylation of the EGF and insulin-like growth factor receptors in parallel with an increase in superoxide and a decrease in hydrogen peroxide accumulation. This decrease in hydrogen peroxide was proposed to reduce the inactivation of the protein tyrosine phosphatase PTP1B, thereby inhibiting growth factor-stimulated receptor phosphorylation and attenuating downstream activation of proliferation pathways involving ERK. This is a potentially important mechanism for the action of copper chelators (Michelle et al., 2009).

**Copper and Anti-cancer drug**

Metal-based antitumor drugs play a relevant role in anti-tumoral chemotherapy. Therefore, in recent years there has been a rapid expansion in research and development of novel metal-based anticancer drugs to improve clinical effectiveness, to reduce general toxicity and to broaden the spectrum of activity. The variety of metal ion functions in biology has stimulated the development of new metallodrugs with the aim to obtain compounds acting via alternative mechanisms of action. Copper complexes are potentially attractive as anticancer agents. Actually, since many years a lot of researches have actively investigated copper compounds based on the assumption proposal that endogenous metals may be less toxic (Marzano et al., 2009).

The mechanism which transports copper throughout the body may also be responsible for the transfer of some chemotherapy drug like Methotrexate, 5-Florouracil. By studying how copper is transported throughout the body, researchers may also gain a better understanding of how these drugs were transported into the cell (Maya et al., 2010). In fact, there is some experimental evidence to suggest that copper complexes can cause established tumor cells to redifferentiate into normal cells, and because of this it has been suggested that, "the future use of copper complexes to treat neoplastic diseases has some exciting possibilities” (Sorenson, 1989).
Reactive oxygen species

Reactive oxygen species (ROS) are essential for life because of their role in many vital processes such as signal transduction and the ability of phagocytes to carry out their bactericidal activity. ROS include free radicals, such as hydroxyl and superoxide radicals, which are substances with one or more orbital electrons with unpaired spin states, and non radicals, including hydrogen peroxide and singlet oxygen. Although carefully controlled processes regulate the production of ROS for their essential functions, many cellular processes result in the generation of ROS. An important site of this nonessential generation of ROS, which constitutes oxidative stress, is the electron transport system that resides within the inner membrane of mitochondria. Normally, electrons are transferred from complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) to coenzyme Q10 and then to complex III, cytochrome c, and complex IV. Finally, 4 electrons are transferred to oxygen with the formation of water. In the process, coupling of electron transport to oxidative phosphorylation results in adenosine triphosphate generation. Although this process is very efficient, about 2% of the electrons escape the electron transport system and react with molecular oxygen to form superoxide radicals. Although superoxide is not highly toxic, mitochondrial superoxide dismutase generates hydrogen peroxide from superoxide radicals, and, in the presence of reduced iron or copper, the highly toxic hydroxyl radical is formed via Fenton or Haber-Weiss reactions (Kenneth, 2004).

The cytochrome P450 monooxygenase system of the hepatic endoplasmic reticulum (microsomes) also generates a substantial amount of ROS in the process of metabolizing a chemically diverse group of compounds that includes most of the drugs that we administer as well as environmental substances. The plasma and nuclear membranes are less active in generating ROS (Kenneth, 2004).

Primary ROS is superoxide, which is formed by the one-electron reduction of molecular oxygen (eqn 1). This is the reaction catalysed by NADPH oxidase (eqn 2, and see below), with electrons supplied by NADPH (Xiao et al., 2002).

\[
O_2 + e^- \rightarrow O_2^- \text{ (superoxide)} \quad (1)
\]
2O₂ + NADPH → 2O₂⁻ + NADP⁺ + H⁺ (2)

Further reduction of oxygen produces hydrogen peroxide. This can arise from the dismutation of superoxide (eqn 3), which can occur spontaneously, especially at low pH:

2O₂⁻ + 2H⁺ → H₂O₂ + O₂ (3)

However, this reaction can also be catalysed by a family of enzymes known as superoxide dismutase (SOD). Therefore, under physiological conditions, once superoxide is formed the presence of hydrogen peroxide becomes almost inevitable. Further reactions may lead to the formation of hydroxyl radicals (•OH), especially in the presence of metal ions through the Fenton or Haber-Weiss reactions. Hydroxyl radicals are extremely reactive, with a short half-life, and will probably react with the first molecule they encounter (Sundaresan et al., 1995). However, in case of In-vitro the hydrated electrons formed by ionizing radiation can reduce it to O₂⁻. O₂⁻ can dismutate to H₂O₂ with the possibility of extra •OH production by metal-catalyzed Fenton reaction (Groen et al., 2005).

HO' + H₂O₂ → H₂O + O₂⁻ + H⁺ (4)

O₂⁻ + H⁺ + H₂O₂ → O₂ + HO' + H₂O (5)

The chain reactions 2 and 3 were originally proposed by Haber and Willstätter in a paper on radical reactions in organic chemistry and biochemistry, and specifically to explain the action of catalase. The opinion of Haber and Willstätter was that enzymes initiated radical reactions, after which the chain reactions consumed the substrate. Iron or other transition metals may just act in the same way (Haber and Weiss, 1932).

The Fenton reaction initiates the chain, Reaction 1,

Fe²⁺ + H₂O₂ → Fe³⁺ + HO' + HO' (1)

Which is then followed by chain Reactions 2 and 3,

HO' + H₂O₂ → H₂O + O₂⁻ + H⁺ (2)
O$_2^-$ + H$^+$ + H$_2$O$_2$ → O$_2$ + HO$^-$ + H$_2$O (3)

While chain termination is caused by Reaction 4:

Fe$^{2+}$ + HO$^-$ + H$^+$ → Fe$^{3+}$ + H$_2$O (4)

At moderate concentrations superoxide anion and related reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes (Bae et al., 1997). Superoxide anions generated extracellularly by transformed cells participate in intercellular signalling and at the same time determine transformed cells as selective targets for intercellular induction of apoptosis. Thus, selective apoptosis induction seems to be based on superoxide anion production by transformed cells, their spontaneous dismutation to hydrogen peroxide and HOCl generation by Myeloperoxidase a novel effector cell-derived peroxidase. HOCl then interacts with target cell-derived superoxide anions to yield hydroxyl radicals. Due to the short diffusion pathway of superoxide anions, hydroxyl radical generation is confined to the intimate vicinity of transformed cells (McCullough et al., 2000).

**Impact of ROS on cancer cells**

Several studies have suggested that ROS have a cell signaling role in many biological systems, both in animals and in plants (Bae et al., 2000). ROS generated by photosensitizers have also been increasingly acknowledged to control signal transduction via the activation of MAPKs, including ERK1/2, ERK5, JNK, and p38 MAPK. It was reported that hydrogen peroxide stimulated MAPK-mediated cell proliferation, activation of MAPKs coincided with the formation of superoxide anion, and superoxide anion increased MAPK-mediated cell proliferation. There is evidence that antioxidants can attenuate MAPK activation. Recently, ROS was found to activate ERK, JNK, and p38 MAPK signaling pathways, but the role of these MAPK in ROS signaling remains obscure (Bae et al., 2000). Further role of ROS in cell proliferation can be understood from several studies showing that tyrosine kinase receptors are involved in signaling via ROS-dependent mechanisms (Sundaresan et al., 1995; Bae et al., 1997).
Greater oxidative stress

Oxidative damage levels rise. Release of transition metal ions that catalyze free-radical reactions is an early stage in oxidative damage. Some metals may bind to DNA to make it a target of damage by peroxides involving site-specific 'OH formation. Abnormally-large rise in "free" Ca\(^{2+}\) occur due to oxidative damage to the ion transporters that normally keep it low.

Activation of transcription factors Adaptive response increasing levels of protective systems (e.g. chaperones, antioxidant enzymes, HO-1, ferritin to sequester iron) that render cell more resistant to subsequent insults. Cell cycle halts to allow repair of DNA damage.

More oxidative damage-mitochondrial damage by ROS and by excessive free Ca\(^{2+}\) can lead to mitochondrial permeability transition and/or cytochrome c release. Excessive DNA damage halts the cell cycle (e.g. via p 53 protein) or initiates apoptosis.

Fig. 4. Schematic representation of cells responding to increase in ROS generation (Bore, 2004 with slight modification)
Synergistic effect of radiation on cancer cells

Radiation therapy has been used in cancer treatment for many decades; it is used to eradicate cancer and help to relieve pain associated with metastases (Bore, 2004). It was also reported that photosensitizers may regulate cellular radiosensitivity by the oxidative signals generated on cell membrane. Increased in the level of ROS by photosensitizer may also be associated with the activation of signaling molecules like PKC, transcription factors like NF-κB. It may also destabilized mitochondrial membrane inducing the release of apoptosis inducing agents like cytochrome c.

Radiation in combination with photosensitizer drugs leads to mitotic cell death in dividing cells and activates pathways that lead to death by apoptosis in interphase cells and differentiated cells. The release of cytochrome c, lead to an activation of the caspase cascade, with caspase 3, leading cells to their death via apoptosis. Alternately, apoptosis proceeds through a mitochondrial-independent pathway, with the ligation of death receptors CD95 (Fas/Apo1/) and the subsequent recruitment of caspases (Shao et al., 2008).

It has been found that cytotoxic effect was induced by initiating oxidative damage to membrane and by triggering intracellular generation of reactive oxygen species (ROS) by gamma radiation in combination with photosensitizer like TPL, EA and TOS in tumor cell line Ehrlich Ascites (EAC), Human cervical (HeLa) and breast (MCF-7) cells (Pandey et al., 2002).

However, it was also reported that gamma radiation may result in a division delay which may have several components including a G1 block, a G2 arrest or an S phase delay. The G1 arrest is absent in many cell lines, and the S phase delay is typically seen following relatively high doses (> 5 Gy). The G1 arrest is regulated by the p53 tumor suppressor gene product. Irradiation results in increased expression of p53, which in turn induces a 21 kDa protein, WAF 1/Cip 1, which inhibits cyclin CDK kinases, thereby resulting in an arrest in G1 stage of cell cycle. In contrast, the G2 arrest is seen in virtually all eukaryotic cells and can occur following high or low doses, even under 1 Gy. The mechanism underlying the G2 arrest may involve suppression of cyclin B1 mRNA in some cell lines and tyrosine phosphorylation of p34cdc2 in others. Similar mechanisms are likely to be operative in the G2 arrest.
induced by various chemotherapeutic drugs like methotrexate and 5-fluorouracil (Maity et al., 1994).

**Chemotherapeutic drugs as photosensitizer**

Photodynamic killing of cancer cells utilizes light in the combination with a photosensitizer (drug) to induce phototoxic reactions. The general mechanism of phototoxic reaction starts by absorption of photon(s) by a photosensitizer followed by intersystem crossing from the excited singlet state to the low-lying triplet state. A triplet photosensitizer can attack target species directly via redox or free radical reaction or transfer its energy) to an oxygen molecule (Bae et al., 2000).

Studies in a variety of cell types have suggested that cancer chemotherapy drugs induce tumor cell apoptosis in part by generating reactive oxygen species (ROS) on exposure to light. Human leukaemia cells with high ROS levels are more sensitive to 2-methoxyestradiol than normal cells. Methoxyestradiol appears to increase oxidative stress in cell, although exactly how it works is not clear. Most agents used in chemotherapy act in specific ways, e.g. cytosine arabinoside by directly interfering with DNA replication, taxol by blocking mitotic spindle formation, methotrexate by interfering with the supply of DNA precursors, irinotecan and the anthracyclines by inhibiting topoisomerases (among other mechanisms), farnesyltransferase interfering with the action of small GTPases, adaphostin by inhibiting protein kinases, and proteasome inhibitors by interfering with cell division. Many and possibly all of these agents are photosensitizer thus expected to cause oxidative stress (Bae et al., 2000).

Evidence was also supported by the study which was conducted on human B lymphoma cells as the targets, and it was found that apoptosis can be induced in the absence of any detectable oxidative stress. Apoptosis was induced with the chemotherapy drugs VP-16. Moreover, chemotherapeutic drug which possess photosensitizer properties are able to induce extensive apoptosis in the absence of any detectable protein or lipid oxidation, measured in both the cytosolic and mitochondrial compartments of the cell (Conklin, 2004).
Oxidative stress generated by photosensitizer during chemotherapy may induce lipid peroxidation thus generating numerous electrophilic aldehydes that can attack many cellular targets (Sentürker et al., 2002). The effects of oxidative stress on cell proliferation are most likely attributable to inhibition of critical enzymes by these aldehydes. The cyclin-dependent kinases (CDKs) are other likely targets of the aldehydes. These enzymes ensure an ordered progression through the phases of the cell cycle, and their inhibition, for example, by the CDK inhibitor p21 (the mediator of the p53 tumor suppressor gene), prevents passage through the restriction point and causes checkpoint arrest subsequently leading to inhibition in cell cycle progression (Hauptlorenz et al., 1985; Dostal et al., 1974; Khoschsorur et al., 1981). Thus, photosensitizer, by reducing the level of aldehydes, may facilitate drug-induced apoptosis via the CD95 pathway (Ashkenazi and Dixit, 1998).

**Photodynamic Therapy**

Photodynamic therapy (PDT) is a systemic treatment used in oncology by a variety of specialists to eradicate premalignant and early-stage cancer and reduce the tumour size in end-stage cancers. In PDT, a photosensitizing agent is used as well as a focal light source and oxygen to selectively destroy cancer cells through photodynamic reaction. Photosensitizing agents are drugs that become active when light of a certain wavelength is directed onto the anatomical area where they are concentrated. The photosensitizing agent is preferentially taken up into and by cancer cells. On exposure to light the photosensitizer expressed in the skin (dermis) is excited to a higher energy state. The transfer of energy to oxygen under these circumstances generates reactive oxygen species which induces cell death. The reactive oxygen species are localized to the cancer cells selectively destroying them and leave the surrounding normal tissue with minimum damage (Vlasta et al., 2007).

PDT is a 3-step procedure.

- In the first step the photosensitizing drug is applied to the lesion, waited for period of time, usually between 30 min-3 hrs, to allow the drug to concentrate in the target cells. The skin may be gently scraped (curretage) before hand to increase the amount of the drug absorbed.
The second step involves activation of the photosensitizer in the presence of oxygen with a specific wavelength of light directed toward the target tissue. Treatment usually lasts between 5-45 minutes. Depending on the type of lesion being treated and the photosensitizing chemical used, a second treatment may be required.

A sunburn reaction develops, which is the third stage, and represents the cytotoxic damage to cells. This usually heals within 4-8 weeks.

Because the light source is directly targeted on the lesional tissue and the photosensitizing drug is preferentially absorbed by malignant cells, PDT achieves dual selectivity, minimizing damage to adjacent skin (Klaus et al., 2008).

Current evidence is showing PDT to be effective in treating Actinic Keratoses (AKs) on the face and scalp, and superficial Basal Cell Carcinomas (BCC). It appears to be as effective as conventional treatments such as cryotherapy (liquid nitrogen), curettage, radiotherapy and topical 5-fluorouracil (Altshuler et al., 2001).
Fig. 5. Systemic photodynamic therapy (Judit and Orsolya, 2005)
RBCs and Anticancer drugs

Erythrocytes are the most abundant cells in the human body, have potential and capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess long circulation half lives and can be loaded with a variety of biologically active compounds using various chemical and physical methods (Herlinde et al., 2003).

The shape of a normal human red blood cell (RBC) under resting conditions is biconcave discocyte. However, RBCs can easily undergo transformation to other shapes with stomatocytes and echinocytes as extremes. Various anticancer agents, generally reactive and labile substances, e.g., oxazaphosphorines and fluoropyrimidines, can induce severe deformation of shape. Shape changes in erythrocytes can induce rheological disturbances which occasionally have pathophysiological consequences. It is difficult to estimate the impact of shape changes on the in vivo behavior of agents of biological interest. However, it has been demonstrated for various anticancer agents that erythrocytes fulfill an important role in their uptake, transport, and release. Moreover, some anticancer agents are capable of influencing important transporters such as MRP and GLUT-1. Monitoring erythrocyte concentrations of certain cytotoxic agents is therefore of interest as the data generated can have a predictive outcome for therapeutic efficacy. This is true for some anticancer drugs which are photosensitizers like cyclophosphamide, Methotrexate, ifosfamide, 5-fluorouracil, lometrexol, 6-mercaptopurine, MRP and GLUT-1 mediated agents (Schrijvers, 2003).

Some recent studies have suggested that increasing reactive oxygen species generation over an established threshold by lowering antioxidant defenses may contribute to selective killing of cancer cells. Such a mechanism for the cytotoxic action of these compounds against cancer cells could involve mobilization of endogenous copper ions, possibly chromatin bound copper and the consequent enhancement in the prooxidant action of the drug. When cancer cell are subjected to chemophototherapy the hydroxyl radicals generated in the vicinity of bound copper in DNA cause strand scission and probably activate apoptosis there by helping in their elimination. This view is also supported by literature where they have suggested that
the role of ROS is to work as secondary death markers for cells that are in the process of committing apoptosis or undergoing necrotic death (Valko et al., 2004). Thus, we hypothesized that during chemophototherapy mobilization of endogenous copper may probably leads to enhancement in generation of oxidative stress which possibly attribute to cytotoxic death of cancer cells.