CHAPTER 1

REVIEW OF LITERATURE

1.1 CANCER

Cancer is becoming an increasingly important factor in the global burden of disease. The estimated number of new cases annually is expected to rise from 10 million in 2000 to 15 million by 2020. Some 60% of these cases will occur in the less developed parts of the world. More than 7 million people now die each year from cancer. Yet with the existing knowledge, at least one-third of cancer cases that occur annually throughout the world could be prevented. Cancer was estimated to account for about 7 million deaths (12% of all deaths) worldwide in 2000 (WHO, 2001), only proceeded by cardiovascular diseases (30% of all deaths), and by infectious and parasitic diseases (19%). Cancer was also estimated to account for almost 6% of the entire global burden of disease in that same year (WHO, 2001). More than 70% of all cancer deaths occurred in low- and middle-income countries and, although the risk of developing or dying from it is still higher in the developed regions of the world, the control of communicable diseases as well as the ageing of the population in developing countries, point to an increasing burden of cancer worldwide. In fact, Pisani et al. (1999) have projected a 30% increase in the number of cancer deaths in developed countries, and more than twice this amount (71%), in developing countries, between 1990 and 2010, due to demographic changes alone. Rising incidence will only add to this burden.

Attempts have been made to quantify the global burden of cancer, and estimate site-specific cancer mortality and morbidity (Parkin, 1998; Parkin et al., 1999). Such studies are of considerable importance in helping to better allocate resources towards the prevention and treatment of cancer. In the early 1980's, Doll and Peto (1981) were already calling attention to the evidence about the avoidability of cancer. According to these authors, approximately 75% of the cases of cancer in most parts of the US, in 1970, could have been avoided. More recently, Parkin et al. (1999) have estimated that there would have been 22.5% fewer cases of cancers in the developing world in 1990, if infections with hepatitis B virus, hepatitis C virus, human papillomaviruses, EBV, HTLV-I, HIV, helicobacter pylori, schistossoma, and liver flukes had been prevented. Another estimate suggests that 230,000 deaths (4.4% of all cancer deaths) from liver cancer could have been avoided with only immunization against hepatitis B (Pisani et al., 1999). According to

Murray and Lopez (1996), cancer of the trachea, bronchus and lung was the tenth leading cause of death in the world in 1990, being the third in the developed regions. Smoking was estimated to be responsible for another 20% of all cancer deaths, all of which are preventable (Pisani et al., 1999). While the need for reliable estimates of cancer burden is clear, much more work is still needed to improve their reliability. Parallel to the development of national systems of death registration, there is a need to develop new methodologies to help improve the accuracy of the current estimates, based on existing data.

1.1.1 Magnitude of cancer problem

The global cancer burden in terms of the annual incidence rate and numbers of new cases of 25 different cancers has been estimated for the year 1990 in 23 areas of the world by Parkin et al., (1999). According to these estimates, the total number of new cancer cases (excluding non-melanoma skin cancer) was 8.1 million, just over half of which occur in the developing countries. Of the total 8.1 million new cases, 4.3 million were men and 3.8 million were women with male to female sex ratio of 1:0.88. Out of these 3.8 million cases in women, 1.88 million cases were estimated to be from developed regions and 1.91 million cases were from developing regions. Cancer of the lung was the most predominant site of cancer in the world in 1990 with 1.04 million new cases, and accounted for 17.9% of all cancer in men and 7% of all cancer in women. These estimates represent an increase of 16% over 1985 estimates with an increase of 14% in men and 21% in women.

Stomach cancer was the second most common cancer in men and accounted for about 12% of all cancer in men. Among women it ranks fourth, accounting for about 8% of all cancers in women. Together in men and women stomach cancer accounted for 10% of the global burden. Thirty-eight percent of these cases occur in china, where stomach cancer remains most common in both sexes, as was the case in Eastern Asia.

Breast cancer was the commonest cancer among women globally, accounting for 21% of all cancers in women. The total number of new cases was estimated to be 795,600 in 1990, and ranked third overall when both sexes were considered together. Colorectal cancer was the second

commonest malignancy in women next to the breast cancer and accounted for 10% of all cancers in women globally. It ranked third in men with 9.4% of all cancers in men. But when cancers were classified in developed and developing regions, colorectal cancer was the second most common cancer in developed countries. As per the estimates of cancer incidence and mortality there were an estimated 2.6 million new cases of cancer in Europe in 1995, representing over 1 quarter of the world burden of cancer. Together with cancers of the colon and rectum, and female breast, the three cancers represented approximately 40% of the new cases in Europe. In men the most common primary sites of all cancer cases were lung (22%), colon and rectum (12%) and prostrate and in females breast (26%), colon and rectum (14%), stomach (7%). Lung cancer was the most common cause of death from cancer in men (29%) and breast cancer was the most common cause of death in females (17%)(Bray et al., 2002).

1.1.2 Descriptive epidemiology of breast cancer

Breast cancer continues to be a major public health problem in all the developed countries and the incidence rate are raising even in the developing countries. The estimated number of new breast cancer cases in 1990 was 795,600, of which 471,500 cases were from developed regions and 324,100 cases were from developing regions. Breast cancer was by far the most frequent cancer among women and accounted for 21% of all cancer in women and ranked third overall when both sexes were considered together. It is the most common cancer among women in all the developed regions (except for Japan, where it was the third ranking cancer after stomach and colorectal cancer), as well as in Northern Africa, South America, East, Southeast and Western Asia. The estimated numbers of breast cancer cases in 1990 was about 11% more than the estimated numbers (719,000) in 1985 (Parkin et al., 1999).

Breast cancer has a major impact on the health of women. Approximately 183,000 women are diagnosed with invasive breast cancer each year and nearly 41,000 women die of breast cancer on United States. In American women, breast cancer was the most frequently diagnosed cancer and the second leading cause of death (Winer et al., 2001). Breast cancer trends in United States have shown increasing rate of new cases with a

sharp rise in the 1980's because of the increased use of mammography. Over the past 30 years, specifically in the decades from 1970-1990, breast cancer patient have increased by 117%, whereas deaths have increased by nearly 50%. These increases were partly due to increase in the population of older women and increase in the average length of life, in addition to screening programmes. Regarding age specific trends, the increase in the incidence rate was nearly 40% for women aged 65 years and older, whereas the increase was less than 5% for women younger than 50 years. Based on the trends to date, there is every reason to believe that the burden of breast cancer will continue to grow not only in terms of the absolute numbers of cases but also in terms of incidence and mortality rates (Sondik, 1994).

1.2 Free Radicals and Carcinogenesis

Free radical can be defined as chemical species possessing an unpaired electron, which is formed by hemolytic cleavages of a covalent bond of molecule, by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when oxygen is partially reduced it becomes, 'activated' and reacts readily with a variety of bio-molecules. This partial reduction occurs in one-electron steps, by addition of one, two and four electron to O_2, which leads to successive formation of reactive oxygen species (ROS). These are five possible species: Superoxide radical ($O_2^{\bullet-}$), Hydroperoxyl radical ($HO_2^{\bullet}$), Peroxide ion (HO_2^-), Hydrogen peroxide (H_2O_2) and Hydroxyl radical (•OH) (Green and Hill, 1984; Chessman and Slater, 1993).

$$O_2 \xrightarrow{e^-} O_2^{\bullet-}$$

$$O_2 \xrightarrow[2H^+]{2e^-} H_2O_2$$

$$H_2O_2 \xrightarrow{Fe^{2+}/Cu^+} \bullet OH + OH^-$$

The $O_2^{\bullet-}$and H_2O_2 so formed, in presence of metal catalyst such as Cu^+/Fe^{2+}, may lead to the formation of most reactive •OH (Halliwell and Gutteridge, 1990). Superoxide radical ($O_2^{\bullet-}$) is reduced to H_2O_2 by the catalytic activity of superoxide dismutase (SOD). Another main enzymatic antioxidants, namely glutathione peroxidase (GPx) and catalase (CAT) again convert H_2O_2 into H_2O. However, accumulation of $O_2^{\bullet-}$ and H_2O_2 results in the formation of •OH, which oxidizes lipids giving rise to lipid peroxidation (LPO) (Chessman and Slater, 1993). Hydrogen peroxide is known to cause DNA breaks in intact cells and purified DNA (Imlay et al., 1988). Malondialdehyde (MDA), which is a major end product and an index of LPO, cross links DNA and protein, and nucleotides on the same and opposite strands (Flohe et al., 1985; Summerfield and Tappel, 1983). It is documented that MDA is mutagenic in mammalian systems, which readily reacts with deoxynucleosides to produce adducts (Marnett, 1994). Products of LPO may cause DNA damage (Halliwell and Gutteridge, 1989; Esterbauer, 1990). Lipid peroxidation may play indirect role in the conversion of procarcinogens to the ultimate carcinogens (O'Brien, 1994). It can cause in the formation cyclic DNA adduct (Bartsch, 1996). Hydroxyl radical also interacts with DNA and causes many types of oxidized nucleosides. 8-hydroxy-2- deoxyguanosine (8-OhdG) is one of the most commonly occurring products of these DNA modifications (Kasai, 1997).

1.2.1 Reactive Oxygen Species (ROS)

1.2.1.1 Superoxide anions ($O_2^{\bullet-}$)

Superoxide anion is the first reduction product of oxygen (O_2) It is a base with the equilibrium with its conjugate acid, the hydroperoxyl radical ($HO_2^{\bullet}$), whose pKa is 4.8. In aqueous solution, at neutral or slightly acid pH, $O_2^{\bullet-}$ is a relatively non-reactive species and dismutates to H_2O_2. This reaction either occurs spontaneously or is catalysed by intracellular enzyme SOD. It has been proposed that $O_2^{\bullet-}$ owing to its unreactivity can diffuse through a long way from its site. At low *p*H in the cell, it becomes protonated ($HO_2^{\bullet}$) and, hence, reactive. The lifetime of $O_2^{\bullet-}$ in the water cellular environment is approximately 10^{-6}s (Pryor, 1986). Superoxide anion can be produced either

by the univalent reduction of O_2 or the univalent oxidation of H_2O_2. The most important source of $O_2^{\bullet-}$ is oxidative enzymes, among which xanthine oxidase (XO) and NADPH/NADH oxidase are the most effective sources (Cross and Jones, 1991). These enzymes possess flavin or transition metal such as Zn, Cu, Fe, which serve as electron donors (Mohazzab and Woline, 1994). Several oxidative enzymes such as aldehydes oxidase and dihydroorotic dehydrogenase have been shown to produce substantial amount $O_2^{\bullet-}$ (MeCord and Fridovich, 1969). Superoxide anion itself directly affects the activity of catalase and peroxidase (Kono and Fridovich, 1982).

Experimental studies showed that $O_2^{\bullet-}$ directly affected some intracellular enzymes, changing their activities, such as epinephrine and creatine phosphokinase (MeCord and Russell, 1988), lactate dehydrogenase bound NADH (Bielski and Chan, 1973), aconitase, 6- phosphogluconate dehuydratase (Gardner and Friedovich, 1992). Research demonstrated an increase production of $O_2^{\bullet-}$ during the proliferation of endothelial cells (Arnal et al, 1996) and involvement of this species in proliferation of B-lymphocytes (Morikawa and Moridawa, 1996). Superoxide anion is able to cause the oxidation of epinephrine (MeCord and Fridovich, 1969). It may also be capable of initiating the peroxidation of unsaturated lipids (Pederson and Aust, 1973). It is also able to cause the oxidation of thiols. Thus, XO acting on xanthine in the presence of oxygen may cause the cooxidation of cysteine (Misra, 1974). Photochemical of enzymatic generation of $O_2^{\bullet-}$ resulted in an increase in chromosome breakage, rearrangement, and sister chromatid exchanges (SCEs) (Emerit et al., 1982). Thus $O_2^{\bullet-}$ may be one of the possible factors for increased risk of carcinogenesis. Recent study found higher generation of $O_2^{\bullet-}$ in breast cancer patient (Ray et al., 2000).

1.2.1.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is the most stable ROS. This is to say that it is the least reactive and the most readily detected. Hydrogen peroxide may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of $O_2^{\bullet-}$. Hydrogen peroxide is the primary product of the reduction of O_2, by numerous oxidase, such as XO, uricase, D-amino acid oxidase, and α - Hydroxy acid oxidase localized in peroxisome (Oshino et al., 1973). In any

system producing $O_2^{\bullet-}$, substantial amount of H_2O_2 is formed. The H_2O_2 is decomposed, although not readily, to H_2O and O_2, like most peroxide, is very sensitive to decomposition by the species that react with it. The reaction is catalysed by redox-active metal complexes, of which catalase and peroxidase are the most effective exponents. Metals ions have a strong effect on the chemistry of O_2 and its reduction products. Experiments with antioxidant enzymes show that H_2O_2 rather than $O_2^{\bullet-}$ is the more essential species to induce cell injury (Junod, 1987). Other researches also indicated H_2O_2 as the most effective species for cellular injury (Rao et al., 1996). It has been demonstrated that H_2O_2 stimulates proliferation of smooth muscle cells (Nishio and Watanbe, 1997). The well-known Fanton reaction is initiated when Fe^{2+} comes in contact with H_2O_2. Ions of Cu, Co, and Ni can also participate in a similar reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

H_2O_2 also reacts with O_2 to initiate Haber – Weiss reaction producing •OH in presence of Fe^{2+}.

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{2+}} O2 + \bullet OH + OH^-$$

Certain estrogen metabolites such as catecholestrogens are involved in carcinogenesis, where H_2O_2 plays an important role. The most active catecholestrogens are the 4-hydroxy derivatives and results shown that 4-hydorxyestradiol (4-OHE2) at physiological concentrations are capable of exhibiting DNA cleaving activity. The formation of these catecholestrogen induced DNA stands breaks has been associated with the utilization of O_2 and the generation of H_2O_2 (Thibodeau and Paquette, 1999). Hydrogen peroxide exposed to cultured MCF-7 cells has been shown to inhibit binding of estrogen receptor to DNA (Lu et al., 1998). Hydrogen peroxide has been associated with the induction of cancer in animals and has been found to induce molecular damage that leads to the formation of transformed cells *in virto* (Shameberger, 1972). It has also been known to be mutagenic and

carcinogenic (Pryor, 1986). Studies also demonstrated that H_2O_2 stimulated the proliferation of smooth muscle cells (Nishio and Watanbe, 1997). Hydrogen peroxide is believed to be involved in *initiation* and *promotion* of carcinogenesis (Birnboim, 1986). Many reports suggested that H_2O_2 could induce DNA breaks in intact cell and purifies DNA (Imlay et al, 1988). Hydrogen peroxide has been known to cause DNA damage in the form of single strand break (SSBs) and double strand break (DSBs) (Thibodeau and Paquette, 1999), chromosomal aberrations (Sofni and Ischidate, 1984), and sister chromatid exchanges (SCEs)(MacRax and Stich, 1979). The induction of chromosomal aberrations by H_2O_2 was also reported in a retrospective study (Tsuda, 1981). Significantly higher H_2O_2 concentration (Ray et al., 2000) and SCEs (Ray et al., 2001) have been reported in breast cancer patients. The studies found comparatively lower H_2O_2 concentration in stage IV than stage II; however the frequencies of SCEs were found to be higher in stage IV. An experimental study suggested that H_2O_2 could induce higher frequency of SCEs when applied at low concentrations (Schoneich, 1967). Studied by Ray et al. (2000) also suggested that there might be and optimum concentration of H_2O_2 that could induce higher DNA damage and higher frequencies of SCEs in breast cancer.

1.2.1.3 Hydroxyl radical (•OH)

Hydroxyl radical is highly reactive. It can react with practically any molecule present in cells. For this reason it is short lived. This insufficient stability does not allow it to diffuse through the cells. Therefore, it reacts with an organic substrate at the sites or near the sites of its formation. The life span of •OH at 37° C is 10^{-9}s. It does not survive for more than a few collisions after its formation. The reactions of •OH are thus site-specific. Due to such short lifetime, it is very difficult to investigate the •OH by conventional methods (Pryor, 1986). This •OH is produced following the reactions of $O_2^{\bullet-}$ and H_2O_2 in presence of metallic ions such as Fe^{2+}/Cu^{+}. Lipids are very susceptible of •OH attack and initiate LPO (Chessman and Slater, 1993). As a result of interaction of •OH with DNA, formation of many types of oxidized nucleoside has been reported. 8-OHdG is one of the most commonly occurring products of these DNA modifications (Kasai, 1997). Formations of 8-OHdG is thought to be a promutagenic lesion since this

induces G: C to T: A transversion unless repaired prior to replication (Cheng, 1992). Cheng et al. (1992) further reported a negative correlation between the level of 8-OHdG and clinical stages as well as lymph node status of breast cancer. They also believed that because of the increased activities of repair enzymes for damaged DNA in advanced staged breast cancer tissue, DNA adducts may be diluted by DNA replication during rapid cell turnover, which may be the reason for negative correlation between 8-OHdG levels and progression of breast cancer (Matsu et al., 2000). Hydroxyl radical is the most potent among ROS, reacting with a wide range of macromolecules at a high rate constant (Hutchinson, 1985). Hydroxyl radical is known to induce conformational changes in DNA including strand breaks, base modifications, damage to tumor suppressor gene and enhances expression of protooncogenes (Halliwell and Aruoma, 1991; Cerutti et al., 1994). Hydroxyl radical is responsible for DNA damage, high frequency of SCEs (Tsuda, 1981) and LPO (Chessman and Slater, 1993).

1.2.1.4 Malondialdhyde (MDA)

Malondialdehyde is the major reactive aldehyde resulting from the peroxidation of biological membrane polysaturated fatty acid (PUFA) (Vaca, 1988). Malondialdehyde a secondary product of LPO is used as an indicator of tissue damage by a series of chain reactions (Ohkawa, 1979). Malondialdehyde is also a by-product of prostaglandin biosynthesis (Hayaishi and Shumizu, 1982). It reacts with thiobarbituric acid and produce red colored products. It has been proposed that the tumorigenic effect of high dietary fat, rich in PUFA takes place via increased synthesis of prostaglandin (Bruda et al., 1980). It is said to be a product of normal metabolism, and is present in a variety of fat containing foodstuffs (IARC, 1985). Furthermore, the direct interaction of DNA with lipid hydroperoxides produced via a chain reaction and other highly reactive compounds formed during LPO has also been proposed as plausible mechanism for the association between high dietary fat intake and carcinogenesis (Wng and Liehr, 1995). It has also been proposed that the effect of dietary fat on cancer occurs through the activation of procarcinogens to ultimate carcinogens by fat oxidation products such as lipid hydroperoxide (Vaca et al., 1988). Malondialdehyde can modify xanthine oxidoreductase activity

through interaction with XO and xanthine dehydrogenase (XDH)(Cighetti et al., 2001). MDA is mutagenic and genotoxic agent that may contribute to the development of human cancer (Feron et al., 1991). Lipid hydroperoxides may directly induce DNA chain breaking (Conchrane, 1991), and lipid peroxyl and alkoxyl radicals may cause base oxidation in DNA (Park, 1992).

Peroxide and hydroperoxides have also demonstrated tumor-promoting activity *in vivo* (Guyton, 1993). It can result in the formation cyclic DNA adducts (Bartsch, 1996). MDA forms adducts with DNA adenine and cystosine, which contributes to the carcinogenicity and mutagenecity in mammalian cells (Marnett and Tuttle, 1980). Malondialdehyde induces mutations include frameshifts and base – pair substitutions. The frameshifte are G → T transversions and C → T and A →G transitions (Bartsch et al., 1994). Large evidence has suggested that MDA is a mutagen and a potential carcinogen (Moller and Walin, 1998). There are also reports the MDA can form DNA adducts which may be responsible for the development of breast cancer (Wang et al., 1996; Thangaraju et al., 1994; Huang et al., 1999). Higher MDA levels have also been reported in solid tumor and human cell lines (Diplock et al., 1993). Significantly higher plasma MDA levels were found in patients with gastric carcinoma (Choi et al., 1999). Higher plasma MDA level in breast cancer was also found by Ray et al. (2000).

1.2.1.5 Nitric Oxide (NO)

Nitric oxide is an inorganic free radical gas, containing odd number of electrons and can form a covalent link with their molecules by sharing a pair of electrons. It is synthesized by a family of isoenzymes called nitric oxide synthase (NOS) located in various tissues, and plays an active role in free radical and tumor biology (Felley and Bosco, 1998). There are three isoenzymes of NOS. Synthesis of NO from NOS-I and NOS-III is calcium dependent, whereas NOS-II is calcium independent. The calcium dependant, NOSs (I and III) produce a small amount of NO (ρmole), whereas much larger amounts of No (ηmole) are released by NOS- II in cells in response to cytokines, lipid lipopolysaccaride (LPS), immune complexes, endotoxins, some co-factors such as colmodulin, NADPH and tetrahydrobiopterin (Stark and Szurzewski, 1992). Nitric oxide plays a vital role as a cell signaling molecule in vascular, nervous and immune sysrem (Moncada, 1991). It

regulates numerous physiological processes, including neurotransmission, smooth muscle contractibility, platelet reactivity and the cytotoxic activities of immune cells. Prolonged exposure to NO inhibits the activity of number of enzymes such as aconitase, complexes I and II, and cytochrome c oxidase (Clementi, 1998). On the other hand, excessive and upregulated NO synthesis has been implicated as causal or contributing to pathophysiological conditions, including many lethal and debilitating disease (Gross and Wolin, 1995).

Nitric oxide and its derivatives produced in inflamed tissues contribute to carcinogenesis. It is believed that NO plays a duel role in cancer. It is a cytostatic and cytotoxic agent, which causes tumor cell killing when generated at higher concentrations by cytokine activated macrophages and endothelial cells, but comparatively at low concentrations, it promotes tumor growth and metastasis (Jenkins et al., 1995).

Nitric oxide plays an active role in free radical and tumor biology (Tamir and Tannenbaum, 1996). Moreover it may have a role in carcinogenesis by inducing DNA strand breaks (Yoshie and Ohshima, 1998). It has been reported that NO can induce DSB by overlapping SSB in the chromosome (Vamvakas et al., 1997). Nitric oxide exerts direct damages including DNA base deamination, peroxynitirite induced adducts formations and strand breaks in the DNA (Yoshie and Ohshima, 1998). Nitric oxide is known to be a potential mutagen (Arroua et al., 1992). It can bind nonheme iron of ribonucleotide reductase to inhibit DNA synthesis (Lepoivre et al., 1991). Nitric oxide may have a role in carcinogenesis by impairing the tumor suppressor function of p53 (Wang and Liehr, 1995). Ohue et al. (1994) hypothesised that NO may either generate or select for the high frequency of p53 mutations that arise at the transition from adenoma to carcinoma *in situ*. Exact mechanism by which NO affects the p53 function is not clear. However, it is proposed that the p53 DNA binding domain contains several cysteine residues, which play an important role in its DNA binding activity. As NO can modify cysteine residues leading to the formation of disulfide bonds, it can thus affect the biological functions of p53 (Hainaut and Milner, 1995). DNA damage triggers the accumulation of p53 protein (Forrester et al., 1996). Deamination of DNA by NO may represent an important endogenous mechanism of genomic alteration. Nitric oxide mediated

deamination of 5- methylcytosine produces thymine. Hence, the over representations of point mutations in human disorders at methylated CpG sites and the high – frequency of mutations at CpG sites in the p53 tumor suppressor gene in human cancers may reflect the etiological contributions of NO in human carcinogenesis (Greenblatt et al., 1994). Peroxynitrite ($ONOO^-$) produced during the reaction of NO and $O_2^{\bullet-}$ is probably responsible for genetic damage (Wink et al., 1998). The reaction of $O_2^{\bullet-}$ with NO, depending on the relative amounts present, can be 5 times faster than the decomposition of $O_2^{\bullet-}$ by SOD. Peroxynitrite is a potent mutagen that can induce trandversion mutations (mainly G → T) at G-C pairs. McRitchie et al. (1997) have investigated the role of estrogen in the NO pathway. Estrogen has been reported to upregulate endothelial NOS (eNOS) gene expression. A correlation between eNOS expression and estrogen receptor (ER) expression has been reported in several human breast cancer cell lines. In ER- positive cells, it is thought that estradiol may enhance the production of NO, which then acts as a free radical to induce mutations leading to a more malignant phenotype (Zeillinger et al., 1996). Human endothelial cells may be activated or modulated by ROS with the release of NO (Madge and Baydoun, 1994). It has been suggested that NO can stimulate $O_2^{\bullet-}$, H_2O_2, •OH induced LPO (Rubbo et al. 1994). Human erythrocytes posses a NO synthase (NOS) that can be activated by oxidative tress and Ca^{2+} accumulation to produce NO and this activation could be involved in the pathogenesis of toxic anemia in breast cancer patients.

By causing oxidative stress in human erythrocytes with H_2O_2 or by increasing the intracellular calcium a gradual increase in both NO and $ONOO^-$ is observed. Furthermore, it has been shown that erythrocytes from breast cancer are subjected to higher oxidative stress by $ONOO^-$ (100 μmoles), with a consequential increase of membrane rigidity, than erythrocytes from healthy individuals. Such mechanical changes may result in shortening of the lifespan of erythrocytes, a feature of toxic anemia in cancer patient (Deliconstantinos et al., 1995). Research suggested that NO was directly involved in the increased frequency of SCEs (Donovan et al., 1997). Reports are also there with suggests that NO also plays important role in increasing frequencies of micronuclei, and chromosomal aberrations (Gurr et al., 1999). In another report, NO was found to induce micronuclei

(Lin et al., 1998). Increased levels of NOS expression or activity have also been reported in human gynecological (Thomsen et al., 1994) and breast tumors (Thomsen et al., 1995), the increased expression was inversely associated with the differentiations of tumor grade. Rajankova et al. (1997) found that the expression of NOS was more abundant in early (T2) lesions than in advance (T4) ones in gastric cancer specimens. Studies using the well characterized murine K-1735 melanoma system of clones, cell lines, and somatic cell hybrids (between non metastatic and metastatic cells) conclude that not - metastatic cells exhibit high levels of inducible NOS (iNOS), whereas metastatic cells do no (Dong et al., 1994). Ray et al. (2001) found significantly higher concentration of NO in breast cancer cases. Comparatively lower production of NO in stage IV than that of the stage II was observed which may be due to lower expression of iNOS in stage IV than in stage II. Lower NO concentration in stage IV may be also because of L-arginine depleted cells, which produce lower $O_2^{\bullet-}$ together with the production of NO. The $O2^{\bullet-}$ and NO may result in the formation of $OONO^-$, which acts on molecular level (Douki and Cadet, 1996) and thereby resulting in higher SCE frequency in stage IV (Ray et al.2001).

Experimental studies suggested that NO contributes to the destruction of circulating tumor cells (Li et al., 1991). High NO concentrations are required to induce apoptosis in mammalian cells (Nictoera et al., 1997) and low concentrations of NO protect from apoptotic cell death (Tzeng et al., 1997). A high concentration of NO kills not only tumor cells but also the normal cells. However, experimental studies demonstrated that transfection of highly metastatic K-1735 murine melanoma cells (which express low iNOS) with an enzymatically active iNOS expressions vector suppress tumorigenicity and metastasis by inducing high levels of NO production (Xie et al., 1996). The systemic administration of MLV-31362 (with IFN- gamma) induces iNOS gene expression in M5076 hepatic metastases, which in turn resulted in their regression (Xie et al., 1995).

1.3 Oxidative Stress

Oxidative stress is caused by exposure to reactive oxygen intermediates, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2),

and hydroxyl radical (•OH) that can damage proteins, nucleic acids and cell membranes. Increasing evidence suggests that the cumulative damage caused by ROS contributes to numerous diseases (Aruoma and Halliwell, 1998). Recent studies also suggest that the effect of these oxidants are integrally linked to the damage caused by hypochlorous acid (HOCl), and the reactive nitrogen intermediates (NO), Peroxynitrite (HOONO) and Nitrosothiols (RSNO). To counter oxidative stress, cells constitively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them.

1.3.1 Chemotherapy induced Oxidative Stress

Antineoplastic agents have been shown to produce oxidative stress in patients who receive these drugs during cancer chemotherapy (Faber et al., 1995; Weijl et al., 1998; Sangeetha et al., 1990). This is evident by the elevation of LPO products; the reduction of total radical-trapping capacity of blood plasma; the reduction in plasma levels of antioxidants such as vitamin E, vitamin C, and β-carotene and the marked reduction of tissue glutathione levels that occurs during chemotherapy. Those agents that generate high levels of ROS include the anthracyclines (eg, doxorubicin, epirubicin, and daunorubicin), alkylating agents, platinum coordination complexes (eg, cisplatin, carboplatin, and oxaliplatin), epipodophyllotoxins (eg, etoposide and teniposide), and the camptothecins (eg, topotecan and irinotecan). The anthracyclines generate by far the highest levels of oxidative stress. This is due to their ability to divert electrons from the electron transport system (ETS) of cardiac mitochondria, resulting in formation of superoxide radicals in addition to generating ROS at other cellular sites (Gille and Nohl, 1997).

Doxorubicin, the most studied anthracycline, possesses a sugar moiety attached to a tetracycline ring that contains a quinone structure. Disruption of the ETS by doxorubicin can occur only following reduction of the quinone to its semiquinone. Doxorubicin is hydrophilic and, in mitochondria of most cells, it cannot penetrate the inner membrane and be reduced by NADH dehydrogenase , which is located on the inner (matrix) surface. However, the structure of the cardiac mitochondria inner membrane is unique in that it possesses an NADH dehydrogenase on the outer (cytosolic) surface in addition to the matrix NADH dehydrogenase that is

present in mitochondria of all cells (Nohl, 1987). In cardiac cells, doxorubicin can penetrate the outer mitochondrial membrane and enter the cytosol where it is reduced by the cytosolic NADH dehydrogenase. Intermolecular rearrangement results in formation of the lipophilic deoxyaglycone of doxorubicin that penetrates the inner membrane. There it competes with coenzyme Q10 (both are structurally quinones) as an electron acceptor, diverting electrons to molecular oxygen with the formation of superoxide radicals. In contrast to the above groups of antineoplastic agents, taxanes (eg, paclitaxel and docetaxel), vinca alkaloids (eg, vincristine and vinblastine), antimetabolites such as the antifolates, and nucleoside and nucleotide analogues generate only low levels of oxidative stress. However, all antineoplastic agents generate some ROS as they induce apoptosis in cancer cells. This is because one of the pathways of drug-induced apoptosis involves the release of cytochrome c from mitochondria (Kaufmann and Earnshaw, 2000). When this occurs, electrons are diverted from the ETS to oxygen by NADH dehydrogenase and reduced coenzyme Q10, resulting in the formation of superoxide radicals. Oxidative stress interferes with cellular processes (cell cycle progression and drug-induced apoptosis) that are necessary for antineoplastic agents to exert their optimal cytotoxicity on cancer cells, and modest levels of oxidative stress have been shown to reduce the cytotoxicity of anticancer drugs (Shacter et al., 2000; Lee and Shacter, 1999). Thus, the formation of ROS that occurs when anticancer drugs are administered may diminish the effectiveness of the treatment. In addition, since some side effects caused by antineoplastic agents appear to be prevented by certain antioxidants, administering these supplements during chemotherapy may diminish the development of side effects as well as improve the response to therapy. This contention is supported by many preclinical and some clinical studies (Conklin, 2000; Lamson and Brignall, 1999).

1.3.2 Doxorubicin induced oxidative stress

Doxorubicin and other anthracycline anti-tumor antibiotics are a group of glycosidic antibiotics isolated from cultures of *Streptomyces peucetius var. caesieus* in the 1960s (Arcamone et al., 1997; Di Marco et al. 1969). Their discovery represented a major breakthrough in the fight against

human cancers, as the drug was found to be unusually potent in a variety of malignancies. Today, doxorubicin and other anthracycline antibiotics remain as major weapons in the combat of human malignant diseases (Davis and Davis, 1979; Rinehart et al., 1974). Owing to its broad anti-tumor spectrum and high potency, doxorubicin is currently a first-line drug in the chemotherapy of a variety of hematopoietic tumors such as leukemias and solid tumors including breast and ovarian cancers, lymphomas, sarcomas, and gastrointestinal neoplasm (Hortobagyi, 1997). In fact, all malignancies except colon cancer are responsive to doxorubicin therapy (Davis and Davis 1979).

Unfortunately, doxorubicin and other anthracyclines induce a number of side effects. In addition to common side effects of cancer chemotherapy such as nausea, vomiting, alopecia, nasal and oral mucosal ulceration, and hematopoietic depression due to inhibition of dividable cells (Creasey et al., 1976), doxorubicin induces a unique cardiotoxicity leading to drug-resistant congestive cardiac failure (Rinehart et al., 1974; Bristow et al., 1978). Indeed, it has been found that the incidence of cardiac failure increases abruptly in patients receiving more than 500 mg/m2 of doxorubicin, a fact that significantly limits doxorubicin's application as a therapeutic agent.

Over the past three decades, doxorubicin induced cardiotoxicity has been extensively investigated, and various studies have been performed to minimize the cardiac side effects of doxorubicin administration. In clinical studies, researchers have attempted to optimize the administration schedule of doxorubicin. In one study, cardiac toxicity was partly reduced on a weekly therapy instead of the conventional once every 3 week injection (Torti et al., 1983). In contrast, another study suggested that 500 mg/kg is a risk-cumulative dose in patients (Lefrak et al., 1973; Billingham et al., 1978). Thus, it is still uncertain which dosage to use for doxorubicin administration. In terms of potential treatments, various interventions based on reducing the number of free radicals produced by doxorubicin have displayed some encouraging effects, but this research is far from being complete. In the end, doxorubicin induced cardiotoxicity remains an important issue, and the optimal strategy for its prevention has yet to be defined.

1.3.2.1 Effects of doxorubicin on Cardiac Function

The cardiotoxicity of doxorubicin has been subdivided into acute and chronic effects, depending on their occurrence following administration of the drug. Cardiac dysfunction is one of the most important features in both acute and chronic cardiotoxicity. It occurs in humans, animal models, and isolated heart preparations after exposure to doxorubicin.

1.3.2.1.1 Doxorubicin Induced Cardiac Dysfunction in Patients

Acute cardiotoxic effects of doxorubicin include hypotension, tachycardia, pericarditis-myocarditis syndrome, left ventricular dysfunction, and various arrhythmias, which develop within minutes after doxorubicin administration (Bristow et al., 1978; Ferrans, 1978). The hypotensive effect is believed to be the consequence of peripheral vascular dilatation, caused by doxorubicin induced release of histamine and catecholamines (Bristow et al., 1983). The pericarditis-myocarditis syndrome tends to affect patients either with or without a previous history of cardiac disease. Pericarditis can occur alone, but most patients exhibited pericarditis accompanied by significant myocardial dysfunction; in some cases, these patients died of cardiogenic shock (Starkebaum and Durack, 1975).

In contrast to the acute cardiotoxic effects of doxorubicin, chronic doxorubicin cardiotoxicity develops several weeks or months after treatment, sometimes even having its onset after the course of therapy has been completed. These delayed effects manifest as cardiomyopathy or as an insidious onset of congestive cardiac failure (Henderson et al., 1978). Although some studies have shown that heart failure is directly related to the amount of myocyte damage that can be evaluated by endomyocardial biopsy (Bristow et al., 1978), others have found that cardiac dysfunction is not perfectly proportional to myocardial morphological changes (Isner et al., 1983). Therefore, it is difficult to accurately predict the severity of doxorubicin induced cardiotoxicity based on the endomyocardial biopsy.

There seems to be a relationship between the occurrence of cardiac failure and the cumulative dose of doxorubicin. When patients receive cumulative doses of doxorubicin higher than 500 mg/m^2, the incidence of cardiac failure reaches 30-50% (Lefrak et al., 1973). Certain changes in the drug administration schedule, such as reducing the acute dose and

increasing injection frequency (thus keeping the cumulative dose unchanged), have been shown to partly suppress the cardiotoxicity of doxorubicin (Torti et al., 1983). This suggests that the development of chronic cardiomyopathy is related to the acute peak drug levels. Thus, acute and chronic cardiotoxicity could be induced by the same mechanism.

1.3.2.1.2 Effects on the In Vivo Cardiac Function of Animals

Cardiotoxicity has been successfully induced in doxorubicin injected animals including mice, rats, rabbits, and dogs (Rosenoff et al., 1975; Doroshow et al., 1979; Unverferth et al., 1985). In one study, doxorubicin injection decreased cardiac output and left ventricular (LV) peak systolic pressure in rats within a few hours by 42% and 36%, respectively (Luo et al., 1997). In another study, systolic and diastolic LV functions were decreased progressively in dogs after multiple injections of doxorubicin (1 mg/kg/week for 8 week, then 1 mg/kg every other week for another 8 week); after 16 week of doxorubicin treatment, maximum systolic dP/dt and cardiac index were decreased by 25% and 40%, respectively, whereas left ventricular end-diastolic pressure (LVEDP) was increased 250% (Unverferth and others, 1985). While diastolic dysfunction may be an earlier sign of doxorubicin-induced cardiotoxicity than systolic dysfunction, high doses of doxorubicin produce both systolic and diastolic dysfunctions. Furthermore, cardiac dysrhythmia is sometimes observed in doxorubicin-treated animals, but it is more difficult to study due to its paroxysmal nature. Doxorubicin induced cardiac dysrhythmia in dogs ranges from acute tachycardia to delayed development of persistent atrial and ventricular ectopic dysrhythmias together with variable depression of atrial-ventricular conductance (Kehoe et al., 1978). The severity of the rhythm changes does not appear to be directly dose-dependent. Overall, doxorubicin-induced cardiac dysfunctions during *in vivo* animal studies are similar to the dysfunction characteristics of doxorubicin-induced cardiotoxicity in patients.

1.3.2.1.3 Effects on Function of Isolated Heart Preparations

doxorubicin impairs cardiac contractility, relaxation, and compliance in isolated animal hearts as a Langendorff preparation at various concentrations (Pelikan et al., 1986; Chen et al., 1987; Pouna et al., 1996;

Platel et al., 1999). The magnitude of cardiac dysfunction depends on the duration and dose of doxorubicin administration. In isolated rat hearts perfused with 10 μM doxorubicin, left ventricular developed pressure (LVDP) increased slightly at 30 min and steadily decreased thereafter to 76% at 70 min; in contrast, LVEDP increased 5-fold after 70 min of perfusion (Pelikan et al., 1986). Elevated coronary resistance during doxorubicin-perfusion could result in myocardial under-perfusion and dysfunction. In non-paced isolated hearts, perfusion of doxorubicin induces a 30% decline in heart rate after 30-min perfusion (Ganey et al., 1991). Apart from the isolated whole heart, other isolated cardiac preparations have also been used. For instance, in isolated heart papillary muscle preparations, doxorubicin dose-dependently causes suppression of both positive and negative rate of force development (±dP/dt) and increased lipid peroxidation at a dose range of 10 μM to 1 mM, although these doses are much higher than the serum peak levels in doxorubicin-administered patients (Lee et al., 1991). In isolated atria preparations, doxorubicin inhibits both contraction frequency and contractile forces at a dose of 0.1 mM, but overexpression of catalase in mouse hearts greatly attenuates these effects. These studies suggest that free radicals play important roles in doxorubicin-induced cardiac dysfunction (Kang et al., 1996).

The relationship between doxorubicin's effects on isolated cardiac preparations and its clinical cardiotoxicity is unclear, since a single dose of doxorubicin rarely causes heart failure in patients, whereas acute exposure to doxorubicin predictably causes dysfunction of isolated preparations. Furthermore, the concentrations required to initiate dysfunction in isolated heart preparations are 10- to 100-fold higher than plasma concentrations of doxorubicin (0.1 μM) in patients receiving chemotherapy (Benjamin et al., 1977; Pelikan et al. 1986). Nevertheless, doxorubicin-induced dysfunction in isolated heart preparations resembles the cardiac dysfunction of *in vivo* animal studies in many aspects. The isolated heart preparation has provided evidence of free radical generation related to its cardiac dysfunction, and it may provide more insights into doxorubicin-induced cardiotoxicity.

1.3.2.1.4 Myocardial Ultrastructural Damage

The decrease in number of cardiac myocytes has been described in the postmortem of cancer patients administered doxorubicin soon after its

clinical application (Lefrak et al., 1973). Further studies indicate that ultrastructural changes are dose-related as well as time-related and that ultrastructural changes persist even months after doxorubicin administration (Jaenke, 1974). The earliest change following doxorubicin-treatment in the human heart is sarcoplasmic vacuolization, which appears to be due to the swelling of the myocyte tubular system (Unverferth et al., 1981). Subsequently, the vacuolar degeneration spreads to form large spaces in the cytoplasm, eventually leading to destruction of myofibrils and other structures. Mitochondrial damages, such as swelling, vacuolization, and disruption of cristae, have also been observed (Doroshow et al., 1985). Studies of endomyocardial biopsies from patients indicate that pathological damage is progressive (Billingham et al., 1978; Mason et al., 1978).

Early and late cardiac morphological changes have been fully investigated in numerous studies (Lambertenghi-Deliliers et al., 1976; Van Vleet et al., 1980). Mitochondrial damage with focal clumping of cristae, densification or swelling of the matrix, and the development of nucleolar segregation were found in mice 24 h after doxorubicin administration (Merski et al., 1976). A larger number of cells are involved and contain dilation of cisternae, extending to all intracellular compartments within 1 to 2 week. Mitochondrial damage was not found 1 to 3 months after doxorubicin injection; only sarcoplasmic vacuolization, loss of myofibrils and disruption of sarcomeres and intercalated disc existed in a number of cardiac myocytes (Bellini and Solcia, 1985). This is different from alterations in hearts from rats and rabbits, in which mitochondrial damages have been consistently observed following doxorubicin treatment (Jaenke, 1974; Doroshow et al., 1979; Unverferth et al., 1985). The chronic cardiomyopathy may represent the cumulative damages that result from repeated acute drug exposures; however, histopathological findings are almost the same in mice receiving single dose and multiple dose injections (Bellini and Solcia, 1985). Although clinical studies indicate that histopathological damage does not correspond very well to the severity of cardiac dysfunction (Isner et al., 1983), it is still used as a marker of doxorubicin-induced cardiotoxicity. The histopathological damage is believed to result from doxorubicin-generated ROS, since the damage is dramatically attenuated or prevented by co-administration of the antioxidant probucol (Singal et al., 1995), co-

administration of the ion chelator dexrazoxane (Della et al., 1996), or the overexpression of endogenous antioxidants such as catalase, metallothionein, or manganese superoxide dismutase (MnSOD) (Kang et al., 1996; Yen et al., 1999).

1.3.2.1.5 Subcellular Effects of doxorubicin

In addition to ultrastructural alterations, dysfunction of SR and mitochondria occurs in hearts from doxorubicin treated animals. SR releases, sequesters, and stores calcium that determines both systolic and diastolic cardiac function of cardiac myocytes; thus, SR dysfunction leads to pronounced cardiac dysfunction. Mitochondrion is the energy metabolic center of oxidative phosphorylation and ATP synthesis. As such, injury to these organelles will inevitably induce cardiac contractility impairment.

1.3.2.1.6 Effects on Mitochondria

Doxorubicin induced mitochondrial dysfunction plays a central role in its cardiotoxicity. Heart mitochondria isolated from rats treated with doxorubicin for 4 to 8 week show cristae damage, swelling, and decrease of Ca^{2+} loading capacity (Zhou et al., 2001). Doxorubicin also inhibits respiration of isolated mitochondria dose-dependently (Gosalvez et al., 1974). The damage of mitochondrial function is related to the downregulation of adenine nucleotide translocase-1, a protein located in the inner mitochondrial membrane that plays a key role in aerobic energy metabolism and regulation of mitochondrial membrane pore transition in cardiac myocytes (Jeyaseelan et al., 1997). Doxorubicin may also impair the cardiac mitochondrial DNA that encodes some important enzymes in the mitochondria. Studies have shown that doxorubicin induces breaking of the mitochondrial DNA helix and slows down mitochondrial DNA synthesis (Ellis et al., 1987). Mitochondrial DNA deletion, therefore, might occur in the hearts of rats or mice chronically treated with doxorubicin (Adachi et al., 1993; Serrano et al., 1999). The incidence of mitochondrial DNA deletion increases with the dosage and duration of doxorubicin administration, and it can be decreased by co-administration of the antioxidant coenzyme Q10, suggesting that ROS are involved in the DNA deletion.

1.3.2.1.7 Effects on Sarcoplasmic Reticulum

Sarcoplasmic Reticulum (SR) has two primary functional sites: longitudinal tubules and terminal cisternae. Longitudinal tubules sequester and transport Ca^{2+} to terminal cisternae, where Ca^{2+} is stored for subsequent release to the contractile apparatus. The release of Ca^{2+} from the terminal cisternae is itself triggered by an increase in intracellular Ca^{2+}. Chronic doxorubicin treatment impairs SR function (Shadle et al., 2000; Boucek et al., 1987), and such functional impairments are associated with decreased expression of the ryanodine receptor, a calcium induced calcium release channel in cardiac SR (Dodd et al., 1993). Some studies have found that doxorubicin suppresses SR Ca^{2+}ATPase and Ca^{2+} uptake capacity by selectively inhibiting their gene expression. The inhibitory effect of doxorubicin on SR Ca^{2+}ATPase gene transcription is mediated by doxorubicin generated H_2O_2 as well as by the downstream upregulation of Erg-1 gene via ERK mitogen-activated protein kinases (MAPK) (Arai et al., 1998; Arai et al., 2000). Other studies indicate that doxorubicin stimulates Ca^{2+} release from SR and selectively opens Ca^{2+} channels localized in terminal cisternae, but it has no effects on the SR Ca^{2+} pump or the contractile apparatus. This suggests that doxorubicin might decrease cardiac contractility by interfering with Ca^{2+} release (Zorzato et al., 1985). Moreover, enzyme activities of adenyl cyclase and guanyl cyclase were inhibited at high doses in isolated SR preparations, which were believed to be the consequence of free radical generation and lipid peroxidation (Lehotay et al., 1983; Singal and Pierce, 1986). Therefore, doxorubicin might inhibit the gene expression of key enzymes in the SR and disturb Ca^{2+} handling by induction of ROS.

1.3.2.2 Free Radicals and doxorubicin Induced Cardiotoxicity

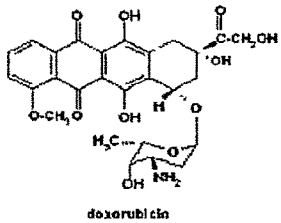
As discussed above, many effects of doxorubicin on the myocardium are related to the formation of ROS. The mechanism of doxorubicin induced cardiac injury has been an active area of investigation in the past three decades. It is believed that the cardiotoxicity of doxorubicin is mediated by mechanisms distinct from those responsible for its anti-tumor effects, such as DNA intercalation and interference with the activities of DNA topoisomerase II (Keizer et al., 1990; Minotti et al., 1999). Several

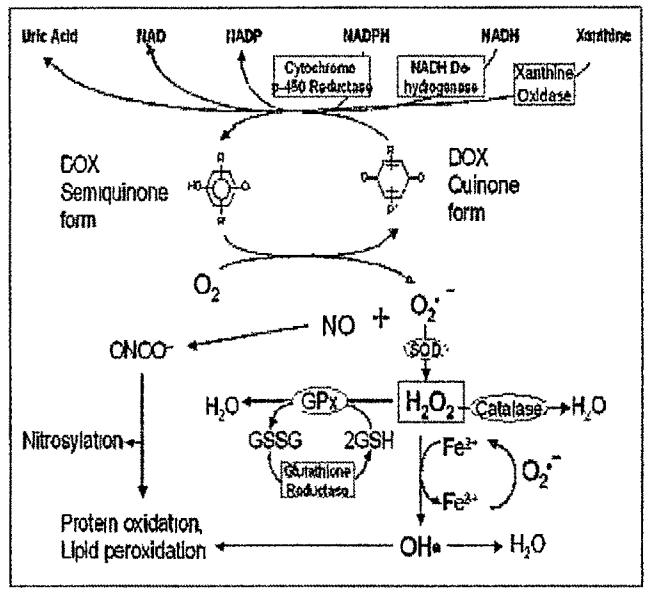
hypotheses have been suggested to explain the acute and chronic cardiotoxicity of doxorubicin. These include formation of free radicals, inhibition of enzymes and proteins, changes in cardiac muscle gene expression, alterations of mitochondrial membrane function, impaired Ca^{2+} handling in SR, mitochondrial DNA damage and dysfunction, and induction of apoptosis (Myers et al., 1977; Kim et al., 1989; Ito, 1990; Arai et al., 1998; Arola et al., 2000; Tokarska-Schlattner et al., 2002). The role of free radicals in doxorubicin induced cardiotoxicity has been extensively studied and is accepted by most researchers.

The free radical generation theory in doxorubicin induced cardiotoxicity is well documented in the literature. Several lines of evidence indicate that doxorubicin generates free radicals via enzymatic pathways in cardiac myocytes. As shown in Figure, doxorubicin is composed of a quinone-form aglycone group and an amino sugar group.The quinone moiety can be reduced to a semiquinone form by single electron donors, such as NADPH, cytochrome P-450 reductase, and NADH and NADH dehydrogenase. This has been shown to occur in liver microsomes, cardiac mitochondria, cultured cardiac myocytes, and rat hearts in numerous studies (Benjamin et al., 1977; Bachur et al., 1979; Doroshow, 1983; Doroshow and Davies, 1986; Davies and Doroshow, 1986; Lee et al., 1991; Sarvazyan, 1996). Once formed, the semiquinone transfers an electron to molecular oxygen, generating a superoxide anion when it cycles back to the quinone form.

Free radicals may also be generated via non-enzymatic mechanisms involving doxorubicin and iron interactions. The Fe^{3+} associates with the ketone and hydroxyl groups of C-11 and C-12 of doxorubicin, followed by an internal redox reaction, wherein an electron flows from the C-14 hydroxyl group to iron, generating a Fe^{2+} doxorubicin free radical complex. This complex reduces oxygen to a superoxide anion, after which an electron flows from Fe^{2+} to doxorubicin, resulting in a Fe^{3+} doxorubicin free radical complex. The complex is then rearranged to Fe^{2+} doxorubicin (aldehyde), which is oxidized to Fe^{3+}doxorubicin (aldehyde) as another molecule of oxygen is reduced to a superoxide anion (Zweier, 1985). Based on this theory, an iron-chelator, dexrazoxane, has been tested and has displayed convincing protective effects in both acute and chronic cardiotoxicity models (Voest et al., 1994; Della et al., 1996).

Doxorubicin Generates Free Radicals





It is believed that the protective effects are due to the interference of dexrazoxane with the doxorubicin-iron complex (Malisza and Hasinoff, 1996). While ROS have been recognized to play important roles in doxorubicin-induced cardiotoxicity, questions have been raised regarding how doxorubicin-generated ROS selectively attack the heart, rather than other organs. Studies in mice indicate that the heart has relatively low levels of endogenous antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), compared to their amounts in the liver (Doroshow et al., 1980). As such, the heart is more vulnerable to doxorubicin-generated ROS insults. Several other factors should not be ignored. First, doxorubicin generated superoxide anions may be converted to hydrogen peroxide by superoxide dismutase. When doxorubicin is incubated with isolated mitochondria from rat hearts, substantial amounts of superoxide anions are formed, accompanying the damage of mitochondrial function (Doroshow, 1983). Superoxide anions may also be converted to more toxic hydroxyl radicals through the Fenton reaction in the presence of Fe^{2+} (Gille and Nohl, 1997).

On the other hand, the peroxynitrite ($ONOO^-$) is believed to be formed from the instantaneous reaction of superoxide anion and nitric oxide, since nitrotyrosine was observed in mouse hearts following doxorubicin treatment (Weinstein et al., 2000). Both hydroxyl radicals and peroxynitrite are highly cytotoxic ROS; they can initiate protein oxidation, LPO and membrane damages in cardiac myocytes. For instance, peroxynitrite results in modification of protein-bound tyrosine to 3-nitrotyrosine, potentially leading to the malfunction of proteins (Beckman and Koppenol, 1996). Studies have shown that lipid peroxidation occurs in doxorubicin-treated hearts. The MDA level was increased in isolated rat LV papillary muscles exposed to 100 μM doxorubicin for 60 min (Lee et al., 1991). The serum creatine phosphokinase (CPK) and cardiac troponin T (cTnT) was elevated in doxorubicin-treated patients and experimental animals as well (O'Brien et al., 1997; Herman et al., 1998; DeAtley et al., 1999). Moreover, free radicals can impair sequestration of Ca^{2+} by the SR. The inhibitory effects were attenuated by incubation with catalase and free radical scavengers, N-acetylcysteine and glutathione (Harris and Doroshow, 1985). The role of ROS in doxorubicin induced cardiotoxicity has been recently further supported by experiments using transgenic technology. Doxorubicin induced cardiotoxicity can be largely suppressed by the overexpression of antioxidant enzymes MnSOD, metallothionein, or catalase in mouse hearts (Yen et al., 1996; Kang et al., 1997; Kang et al., 2002). In summary, ROS play pivotal roles in doxorubicin induced cardiotoxicity, and antioxidants may minimize the cardiac injury induced by doxorubicin generated ROS.

1.3.2.3 Role of endothelial nitric oxide synthase in doxorubicin induced ROS formation

Recent studies revealed that doxorubicin could undergo a direct reduction at the reductase domain of the eNOS, leading to enhanced superoxide formation (Vásquez-Vivar et al., 1997). Other investigators have also addressed the toxicological significance of NOS-mediated myocardial metabolism of doxorubicin (Garner et al., 1999). A recent report by Garner et al. (1999) raised the possibility that NOS is the major enzyme involved in the cardiotoxicity of doxorubicin. It was reported that exposure of endothelial cells to H_2O_2 promotes eNOS expression (Kalivendi et al., 2001). Doxorubicin

induced apoptosis was shown to be linked to intracellular H_2O_2 formation Although inhibitors of eNOS (L-NAME, L-thiocitrulline, etc.) did not significantly affect doxorubicin induced apoptosis, doxorubicin induced toxicity is mediated by intracellular H_2O_2 as well as the calcium influx (Kotamraju et al., 2000; Kalivendi et al., 2001).

Doxorubicin treatment causes an increase in eNOS transcription and protein activity in bovine aortic endothelial cells (Kalivendi et al., 2001) and that pretreatment with antisense eNOS mRNA causes a decrease in doxorubicin induced apoptosis. Antioxidants inhibiting mitochondrial H_2O_2 decreased doxorubicin induced apoptosis. For example, the pretreatment of cells with ebselen (a glutathione peroxidase mimetic) totally abolished doxorubicin induced apoptotic cell death. Therefore, it is likely that H_2O_2 is responsible for initiating the apoptotic signaling. These antioxidants also decreased doxorubicin stimulated eNOS overexpression. Results from this study lead us to conclude that doxorubicin induced apoptosis is linked to the redox activation of doxorubicin by eNOS.

1.3.2.4 Role of iron in doxorubicin toxicity

The role of iron in doxorubicin cardiotoxicity has been a subject of several investigations (Thomas and Aust, 1986; Minotti et al., 1998; Myers et al., 1982; Muindi et al., 1984; Minotti et al., 1999). Early on it was reported that the iron-chelator bispiperazine dione (ICRF-187 or dexrazoxane) protects experimental animals against doxorubicin-mediated cardiotoxicity (Herman et al., 1985). It was later demonstrated that ICRF-187 had a cardioprotective effect in women undergoing doxorubicin chemotherapy for advanced breast cancer (Speyer et al., 1988). ICRF-187 did not appreciably interfere with the antitumor effect of doxorubicin (Curran et al., 1991). ICRF-187 is a pro-drug and becomes an active drug after being hydrolyzed intracellularly to form an EDTA-like bidentate chelator. It was thus proposed that ICRF-187 chelates 'free' intracellular iron and prevents ROS formation (Seifert et al., 1994). Thus, to demonstrate *in vitro* efficacy, cells should be preincubated with this drug to allow adequate time for uptake and intracellular conversion of the pro-drug to an active form. Recent *in vivo* data show that ICRF-187 had to be pre-administered in patients for maximal therapeutic efficacy against doxorubicin toxicity (Tetef et al., 2001). Reports

by Klausner et al., (1993) have revealed a novel link between iron-regulatory protein (IRP-1) and oxidative stress (Martins et al., 1995; Pantopoulos and Hentze, 1995; Pantopoulos et al., 1997; Gehring et al., 1999; Rouault and Klausner, 1996). This involves regulation of the intracellular iron storage protein ferritin and tranferrin receptor (TfR) expression on the cell surface. IRP-1 is a critical regulator of cellular iron metabolism. IRP-1 is a bifunctional protein, alternating between aconitase and an iron-responsive element (IRE) binding activities (Klausner et al., 1993). The presence or absence of the 4Fe-4S clusters determines the function of IRP- 1 as an aconitase or IRE-binding protein. Inactivation or disassembly of the 4Fe-4S cluster occurs by ROS or by oxidant-induced stress-response signaling pathway (Cairo et al., 1996). In other words, IRP-1 senses iron levels by switching between cytoplasmic aconitase and IRP-1 activity. Exposure of cells (e.g. B6 fibroblasts) to extracellular H_2O_2 or intracellular ROS generator (i.e. quinone-containing compounds) induces IRE binding activity, with a concomitant loss of 4Fe-4S clusters and aconitase activity. These changes are accompanied by an increase in TfR expression and decreased ferritin synthesis – conditions that exacerbate cellular oxidative stress. However, induction of heme oxygenase that occurs with time is seen as a compensatory protective mechanism (Vile et al., 1994). How doxorubicin toxicity is controlled by the IRE/IRPI system is a new and exciting twist to an old story surrounding doxorubicin, iron, and ROS (Minotti et al., 1999).

1.3.2.5 Doxorubicin induced apoptotic signaling

Emerging research indicates that apoptosis of cardiomyocytes contributes to the development of heart failure (Narula et al., 1996). Published data indicate that doxorubicin treatment in endothelial cells and myocytes causes apoptotic cell death, as characterized by caspase activation and internucleosomal DNA degradation (Sawyer et al., 1999; Kotamraju et al., 2000; Wang et al., 1998). This novel concept of cardiomyocyte apoptosis has broader implications, especially with regard to myocardial function, because loss of cardiomyocytes could initiate or exacerbate heart failure (Kang and Izumo 2000; Haunstetter and Izumo 1998; Narula et al., 1999). In contrast to the cytostatic mechanism of tumor cell apoptosis (Konopa, 1988), doxorubicin induced myocyte and endothelial apoptosis is mediated by oxy

radical chemistry (Sawyer et al., 1999; Kotamraju et al., 2000). The proapoptotic effect of doxorubicin in myocytes and endothelial cells has been attributed to H_2O_2 formation. On the one hand, doxorubicin induced apoptosis is beneficial in cancer treatment; however, on the other hand, the proapoptotic effect of doxorubicin in myocytes and vascular cells is responsible for its cardiotoxicity. A better understanding of doxorubicin induced pro and antiapoptotic signaling pathways in cancerous and non-cancerous cells may lead to new and improved therapeutic protocols for mitigating the toxic side effects of doxorubicin. In general, a multitude of signaling pathways could be involved in apoptosis, depending on the type of cells and stimulus (Carmody and Cotter, 2001). Apoptosis depends on the activation of caspases (Green and Kroemer, 1998; Green and Reed, 1998). The two main pathways stimulating caspases are: the extrinsic pathway mediated by formation of a cytosolic complex between the cell surface receptor, Fas, and Fadd (receptor-associated death domain) protein and subsequent activation of initiator caspases (e.g. caspase-8); and (ii) intrinsic pathway involving the release of mitochondrial cytochrome *c* (a 13-kDa heme-containing protein) leading caspase-9 activation through formation of apoptosome complex between apoptosis activating factor (Apaf-1) , cytochrome *c*, and pro-caspase-9 .

In healthy cells, Apaf-1 is sequestered from cytochrome *c* by the mitochondrial membrane barrier. In apoptotic cells, mitochondrial membrane potential is impaired, causing opening of permeability transition pore. This, in turn, releases cytochrome *c* present in the mitochondrial intermembrane space into the cytosolic compartment (Kluck et al., 1997). The two pathways are connected via caspase-8 dependent Bid cleavage (Fulda et al., 2001). The Bcl-2 family of proteins regulates the integrity of the outer membrane barrier (Gross et al., 1999). The Bcl-2 (B-cell lymphocyte/ leukemia-2 gene) protein family consists of both anti-apoptotic members (e.g. Bcl-2, Bcl-XL) that block cytochrome *c* release into the cytosol and inhibit apoptosis, and the proapoptotic members (Bid, Bax, Bad, etc.) that induce the release of cytochrome *c* by facilitating mitochondrial pore opening. It has been proposed that the translocation of Bad and Bax from the cytosol to the mitochondria and their interaction with Bcl-2 forming heterodimers (Oltvai et al., 1993) (e.g. Bax/Bcl-2 or Bax/Bax) is a critical

factor in regulating cytochrome *c* release. Recently, investigators have shown that H_2O_2 and $O_2^{\bullet-}$ induce distinctly different apoptotic pathways in cardiomyocytes (von Harsdorf et al., 1999). H_2O_2 but not $O_2^{\bullet-}$ exposure to myocytes promoted translocation of the proapoptotic proteins (Bax, Bad) to the mitochondria, formation of heterodimers with Bcl-2, and the subsequent release of cytochrome *c*. There is increased evidence connecting p53 to intracellular oxidant formation (Wang et al., 2000; Huang et al., 2000). p53 activation may directly induce the activation of Bax gene which contains p53 binding site. As both H_2O_2 and $O_2^{\bullet-}$ induce p53, it is critical to understand how oxidants modulate the translocation of Bax/Bad from the cytosol to the mitochondria. This information is lacking in doxorubicin-induced myocyte and endothelial apoptosis, although several studies probing such mechanisms exist in cancer cells. The signaling pathways induced by doxorubicin appear to be dependent upon the cell type. For example, overexpression of GPx-1 enzyme in cancer cells attenuate doxorubicin-induced apoptosis by modulating sphingolipid (e.g. ceramide) signaling. Ongoing research suggests that overexpression of GPx-1 completely inhibits doxorubicin-induced apoptosis in endothelial cells and myocytes. The role of H_2O_2 in doxorubicin mediated mitochondrial and FAS receptor ceramide dependent still remains to be investigated. doxorubicin is now one of the most frequently used apoptotic signaling agents that induces oxidative stress from intracellular H_2O_2 formation. It is conceivable that doxorubicin will yield new and important mechanistic information on apoptotic signaling pathways in the future.

1.3.3 Cisplatin induced oxidative stress

Cisplatin [cis-diamminedichloroplatinum (II): CDDP] is a widely used cancer chemotherapeutic agent whose clinical use is limited by its renal toxicity, an effect which has been well documented in all species studied to date including mice, rats, dogs and humans (Goldstein and Mayor, 1983). Cisplatin was discovered to have cytotoxic properties in the 1960s, and by the end of the 1970s it had earned a place as the key ingredient in the systemic treatment of germ cell cancers. Since the early seminal work in the preclinical and clinical development of this drug, several thousand analogues have been synthesized and tested for properties that would

enhance the therapeutic index of cisplatin. Although renal toxicity is the dose-limiting factor for the use of cisplatin, other associated toxicities include emesis, nausea, diarrhea, anorexia, hair epilation and myelosuppression (Daly, 1996).

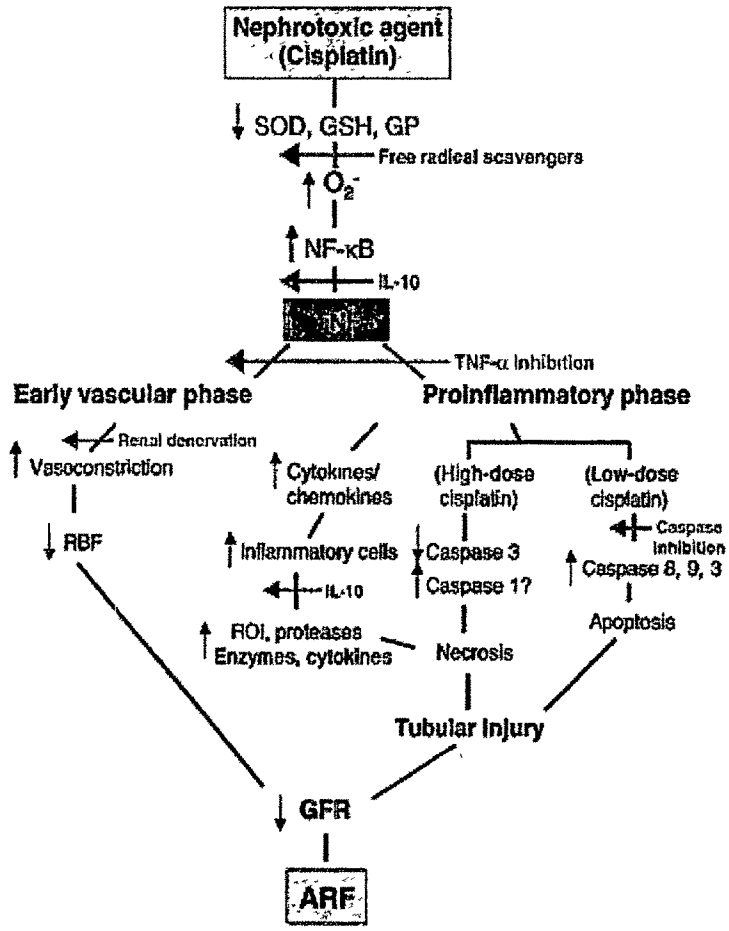
Cisplatin induced nephrotoxicity is not related to the toxicity of platinum per se as has been commonly presumed. Rather it seems that a metabolite of cisplatin, possibly an electrophile such as the aquated and hydroxylated form of cisplatin, mediates its nephrotoxicity. This reactive metabolite of cisplatin may bind covalently to essential macromolecules of the kidney, resulting in nephrotoxicity (Goldstein and Mayor, 1983).

Mechanisms for renal toxicity range from definitive histologic changes found in the proximal convoluted tubules to physiologic and biochemical alterations involving a decrease in mitochondrial respiratory function, enzymic activity in the respiratory chain and glutathione peroxidase, and effects on cellular calcium homeostasis. Important factors related to nephrotoxicity include age, renal irradiation, and concurrent alcohol intake. A potential mechanism influencing the sensitivity of cells to cisplatin may result from the interaction of specific proteins with cisplatin-damaged DNA. Other molecules, including RNA and proteins, also react with cisplatin. These reactions occur either by a direct pathway in which the co-ordinated chlorides are displaced by nucleophilic entering groups or by an indirect pathway in which the entering nucleophiles react with cisplatin molecules that have already exchanged chloride groups for solvent (H_2O).

Cisplatin protein interactions are important in determining the therapeutic efficacy of the antitumor agent. Binding of cisplatin to plasma proteins significantly alters the rate of clearance of the drug from circulation. Reactions of native proteins with cisplatin may be responsible for the observed toxicity to the kidneys and gastrointestinal tract (Daly, 1996).

The effect of cisplatin on liver and kidney functions has been reported in several studies. Treskes et al. (1992), Hanigan et al. (1994) and Bogin et al. (1994) showed that an injection of cisplatin changed liver and kidney enzyme activities. Also, Nair et al. (1991) reported that body weight; haemoglobin levels and leucocyte counts were decreased after cisplatin injection in mice. Renal failure in rats treated with cisplatin could be a result

of the lower enzymatic activities in the kidney, as well as the less efficient oxidative phosphorylation and adenosine triphosphate production of the mitochondria. Administration of cysteine and vitamin E together with cisplatin partially reversed the uraemia and many of the biochemical changes induced by cisplatin.



Biochemical studies with heavy metals show that they react with free sulphydryl (SH) groups. It is postulated that the nephrotoxicity caused by several heavy metals, e.g. mercury, is related to the intracellular decrease of reduced glutathione, enzymes are then inactivated because their SH-groups are not maintained in a reduced form. Levi et al. (1980) showed that following the administration of cisplatin to rats, there was a significant decrease of SH groups per gram-wet weight. This change was seen prior to the elevation of blood urea nitrogen (BUN) and creatinine in the blood,

suggesting that SH group depletion may be a primary event leading to the renal failure. The decrease of SH groups was due to a decrease of protein-bound SH, with the greatest decline in the cytosolic and mitochondrial fractions. Ways of protecting SH groups and reducing the toxic effects caused by cisplatin has been reported (Daly, 1996). Administration of substances containing sulphydryl groups, or with antioxidant properties, before cisplatin, significantly reversed the inhibitory effects caused by cisplatin alone, supporting the hypothesis that the cisplatin toxicity is associated with the reduction of free sulphydryl groups (Daly, 1996).

Studies also suggest that apoptotic cell death may play an important role in the development of cisplatin induced acute renal failure (Zhou et al., 1999). Findings also suggest that the nephrotoxic effects of cisplatin may, in part, be related to protein kinase C (PKC) activation in the renal tubules (Ikeda et al., 1999). Cytokines are synthezised primarily by immune cells and have plieotrophic effects on various cells. It has been shown that the expression of various cytokines and chemokines including TNF-α are upregulated in cisplatin-induced renal failure in mice. TNF-α is known to induce apoptosis in cultured renal proximal tubule cells. Using pharmacological agents, which antagonize the secretion and action of TNF-α, helped to alleviate the severity of cisplatin toxicity as measured by blood urea nitrogen and changes in gene expression of cytokines suggesting the direct role of TNF-α in cisplatin-induced nephrotoxicity. The mechanism of cisplatin-induced upregulation of TNF-α expression and the role of TNF targets in producing nephrotoxicity is being studied.

Previous reports suggest that cisplatin induced nephrotoxicity is by increase in lipid peroxidation (Hanneman and Baumann, 1991) and depletion of cellular thiols (Levi et al., 1980) in kidney tissues following cisplatin treatment. Cisplatin inhibits activities of antioxidant enzymes (SOD and catalase) in rat kidneys suggesting that cisplatin nephrotoxicity results from generation of reactive oxygen species (Sdzuka et al., 1992). Enhancement in lipid peroxidation and decrease in reduced glutathione and antioxidant enzymes (SOD and catalase) contributes to cisplatin-induced nephrotoxicity.

1.4 Defenses against Free Radicals: Antioxidants

Aerobic organisms possess antioxidant defense systems that deal with ROS produced as a consequence of aerobic respiration. Reactive oxygen is related to both, the arrest of growth and the start of cell differentiation. Low concentrations of reactive oxygen intermediates may be beneficial or even indispensable in processes such as intracellular messaging and defense against microorganisms, but higher amounts of active oxygen may be harmful to cells and organisms. Small amounts of ROS, as hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli (Lee et al., 1998). Low levels of ROS may be indispensable in a plethora of processes, including intracellular messaging (Schulze-Osthoff et al., 1997) leading among others to proliferation or apoptosis (Vogt et al., 1998), immunity (Sun et al., 1998), and defense against microorganisms (Lee et al., 1998). In contrast, high doses and inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules (Lledias et al., 1998).

The prevention of lipid peroxidation is an essential process in all the aerobic organisms, as lipid peroxidation products can cause DNA damage. Increased lipid peroxidation and decreased antioxidant protection frequently occurs (Tampo et al., 1998); epoxides may spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA and protein. Such a reaction may lead to cytotoxicity, allergy, mutagenicity and carcinogenicity, depending of the properties of the epoxide in question. Moreover, oxidative events may play an important role in the mechanism of action of ether lipids, and oxidizability may contribute to cellular drug sensitivity (Wagner et al., 1998).

A wide array of enzymatic and non-enzymatic antioxidant defenses exist, including SOD, GPX, CAT, glutathione (GSH), beta-carotene, vitamin A ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) (Mataix et al., 1998). There is an interrelationship between both, the activities, and the intracellular levels of these metabolites, protecting themselves from oxygen toxicity (Grazioli et al., 1998).

1.4.1. Antioxidant Enzymes

1.4.1.1 Superoxide dismutase

Superoxide dismutase destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by CAT or GPX reactions. SOD converts the highly reactive superoxide radical to the less reactive H_2O_2.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Another function of SOD is to protect dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate dehydratase and fumarases A and B) against inactivation by the superoxide radical (Benov and Fridovich, 1998). Four classes of SOD have been identified, containing either a dinuclear Cu, Zn or mononuclear Fe, Mn or Ni cofactors (Whittaker & Whittaker, 1998). Fe-SODs and Mn-SODs show homology and posses identical metal chelating residues at the active site, sharing substantial sequence and three dimensional structural homology, while the other superoxide dismutases are structurally unrelated. In humans, there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular-SOD (Majima et al., 1998). SOD catalyses the dismutation of $O2^-$ by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates (Hsieh et al., 1998).

1.4.1.2 Catalase

Catalase is a tetrameric haeminenzyme consisting of 4 identical tetrahedrally arranged subunits of 60 kDa. Therefore, it contains 4 ferriprotoporphyrin groups per molecule, and its molecular mass is about 240 kDa. Catalase is one of the most efficient enzymes known. It is so efficient that it cannot be saturated by H_2O_2 at any concentration (Lledias et al., 1998). CAT reacts with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol,formic acid, phenol) using 1 mole of peroxide in a kind of peroxidase activity:

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

Hydrogen peroxide is enzymically catabolized in aerobic organism by catalase and several peroxidases. In animals, H_2O_2 is detoxified by CAT and GPX. Catalase protects cells from H_2O_2 generated within them. Even though CAT is not essential for some cells type under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Hunt et al., 1998). The increased sensitivity of transfected enriched catalase cells to adriamycin, bleomycin and paraquat is attributed to the ability of catalase in cells to prevent the drug-induced consumption of O_2. Thus, capturing H_2O_2 before it can escape the cell and converting it to O_2. In this way, catalase can maintain the concentration of O_2 either for repeated rounds of chemical reduction or for direct interaction with the toxin (Speranza et al., 1993).

1.4.1.3 Glutathione peroxidase

The selenium-containing peroxidases, being the more important example glutathione peroxidase, catalyze the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage.

$$ROOH + 2\ GSH \xrightarrow{GPX} ROH + GSSG + H2O$$

There are at least five GPX isoenzymes found in mammals. Although their expression is ubiquitous, the levels of each isoform vary depending on the tissue type. Cytosolic and mitochondrial glutathione peroxidase (cGPX or GPX1) reduces fatty acid hydroperoxides and H_2O_2 at the expense of glutathione. GPX1 and the phospholipid hydroperoxide glutathione peroxidase GPX4 (or PHGPX) are found in most tissues. GPX4 is located in

both the cytosol and the membrane fraction. PHGPX can directly reduce the phospholipid hydroperoxides, fatty acid hydroperoxides, and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins (Imai et al., 1998). GPX1 is predominantly present in erythrocytes, kidney, and liver, and GPX4 is highly expressed in renal epithelial cells and testes. Cytosolic GPX2 (or GPX-G1) and extracellular GPX3 (or GPX-P) are poorly detected in most tissues except for the gastrointestinal tract and kidney, respectively. Recently, a new member, GPX5, expressed specifically in mouse epididymis, is interestingly selenium-independent (De Haan et al., 1998). GPX (80 kDa) contains one selenocysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity (Ding et al., 1998). Although GPX shares the substrate, H_2O_2, with catalase, it alone can react effectively with lipid and other organic hydroperoxides.The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas CAT becomes more significant in protecting against severe oxidant stress (Yan and Harding, 1997). In animals cells, and specially in human erythrocytes, the principal antioxidant enzyme for the detoxification of H_2O_2 has for a long time been considered to be GPX, as catalase has much lower affinity for H_2O_2 than GPX (Izawa et al., 1996). Cells depleted of glutathione peroxidase were more sensitive to the toxicity of paraquat and adriamycin than untransfected parental cells from which they derived but not more sensitive to bleomycin, menadione, or phenazine methosulfate. In fact that the mildly increased sensitivity to paraquat and adriamycin was the consequence of the diminished cellular content of glutathione peroxidase was confirmed by the increase in sensitivity of untransfected cells after treatment with buthionine sulfoximine, an agent which depletes cells of glutathione.

These data strongly suggest that the enzymatic action of GPX protects cells from the toxicity of paraquat and adriamycin. The toxin that these agents engender is likely to be hydrogen peroxide or another hydroperoxide upon which glutathione peroxidase acts (Taylor et al., 1993). GPX equally protects against the oxidation of dihydrorhodamine 123 (an indicator dye) by peroxynitrite, requiring GSH as reductant. Thus, there is also a function of GPX and potentially of other selenoproteins containing selenocysteine or selenomethionine, in the GSH-dependent maintenance of a defense line

against peroxynitritemediated oxidations, as a peroxynitrite reductase (Sies et al., 1997).

1.4.2 Effects of Antioxidants on anticancer drug Induced oxidative stress

A number of free radical scavengers have been shown to protect the heart against doxorubicin induced cardiotoxicity. These include α-tocopherol, N-acetylcysteine, probucol, and dexrazoxane (Wang et al., 1980; Herman et al., 1985; Siveski-Iliskovic et al., 1995; Della et al., 1996; Nazeyrollas et al., 1999). Dexrazoxane, which is the most effective cardioprotective thus far in doxorubicin induced cardiotoxicity, prevents superoxide anion formation by associating with Fe^{2+} and by blocking the Fenton reaction (Malisza and Hasinoff, 1996). Unfortunately, all these compounds have pronounced clinical disadvantages. The protective effects were not consistently observed in doxorubicin induced cardiotoxicity (Breed et al., 1980; Legha et al., 1982; Wang et al., 1980). Probucol, a lipid-lowering antioxidant, confers significant protection against doxorubicin-induced cardiotoxicity (Siveski-Iliskovic et al., 1995); however, concerns about its HDL-lowering property discourage its clinical application. A cytoprotective drug, amifostine, has also been approved by the FDA to protect against doxorubicin-induced cardiotoxicity, but it is less potent than dexrazoxane and does not prevent the mortality and weight loss caused by doxorubicin in spontaneously hypertensive rats (Herman et al., 1994). Dexrazoxane, the only cardioprotective drug currently available clinically, only reduces 50% of doxorubicin-related cardiac complications (Hasinoff, 1998). Moreover, it interferes with the anti-tumor activity of anthracycline antibiotics and potentiates doxorubicin's myelosuppression (Koning et al., 1991; Sehested et al., 1993).

The nephrotoxicity induced by cisplatin in human and experimental animal has been shown to be protected by the prior treatment of various antioxidans like ebselen (Yoshida et al., 2000), vitamin C (Antunes et al., 2000) and selenium (Caffrey and Frenkel, 2000; Antunes et al., 2001). Another antioxidant like Green tea extract, melatonin, lovastatin and resveratrol has recently drawn attention.

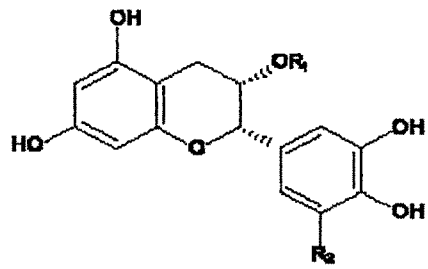
1.4.2.1 Green tea extract

Plants, including herbs and their derivatives, have been used to combat a variety of ailments (Pezzuto, 1997; Challa et al., 1997). The polyphenols present in these botanicals (fruits, vegetables, herbs, etc.) appear to be partially responsible for many of the protective effects of plants against a variety of diseases, including cancer (Kelloff et al., 1996). Several animal studies have demonstrated an anticarcinogenic effect of polyphenols (Yang et al., 1997). Some of the polyphenols studied for their anticarcinogenic potential are flavones, flavonols, isoflavones, and catechins. Tannins, present in many plant foods, have also been shown to possess anticarcinogenic and antimutagenic potentials. (Chung et al., 1998) The lower incidence of certain types of cancer in Mediterranean countries is considered to be associated with that region's high consumption of olive oil, which contains polyphenolic compounds such as hydroxytyrosol and oleuropein (Visioli et al., 1998) Curcumin, a yellow coloring agent present in the spice turmeric, and ellagic acid, a polyphenol abundant in fruits (especially berries), nuts,and vegetables, have been shown to afford protection against chemical carcinogenesis in animals (Stoner and Mukhtar, 1995). Recent studies have shown that resveratrol (3,5,4'-trihydroxystilbene), a polyphenolic antioxidant found in grapes, red wine, berries, and peanuts, exhibits chemopreventive effects in mice. Recently, tea derived from the *Camellia sinensis* plant, which contains many polyphenolic compounds, has also been shown to protect against a variety of ailments, including cancer (Weisburger, 1996).

1.4.2.1.1 Green Tea and Cancer Chemoprevention

The anticarcinogenic and antimutagenic properties of green tea were first elucidated a decade ago (Khan et al., 1988). Since then, several laboratory and epidemiologic studies have been conducted (Kohlmeier et al., 1997). It has been demonstrated in mice that oral consumption or topical application of green tea and its polyphenolic constituents affords protection against carcinogenesis induced by chemicals or ultraviolet radiation (Mukhtar et al., 1994). Polyphenols isolated from green tea or water extract of green tea have been shown in other animal models to afford prevention against chemically induced carcinogenesis in lung, stomach, esophagus,

duodenum, pancreas, liver, breast, and colon (Weisburger et al., 1997). On the basis of some recent studies, it is now believed that much of the cancer chemopreventive effects of green tea are mediated by epigallocatechin gallate (EGCG), which is the major polyphenolic constituent of green tea. One cup of brewed green tea contains up to 200 mg EGCG. A recent bioavailability study showed that frequent consumption of green tea results in high levels of EGCG in various body organs, indicating that green tea consumption might protect against cancers of multiple body sites (Suganuma et al., 1998). The anticancer and anti-inflammatory properties of green tea have interested the food, beverage, and cosmetic industries. Many consumer products, including beverages, foods, health care products, and cosmetics, are now supplemented with extracts of green tea, indicating its human acceptability. Green tea is also believed to possess most, if not all, the qualities of an ideal chemopreventive agent. In addition to imparting preventive and therapeutic effects, green tea has also been shown to modulate and increase the efficacy of cancer chemotherapeutic drugs. Sadzuka et al. (1998) demonstrated recently that the oral administration of green tea resulted in enhanced tumor inhibitory effects of doxorubicin on Ehrlich ascites carcinomas implanted in CDF1 and BDF1 mice. Of interest is that this study showed that green tea treatment resulted in an increase in the level of doxorubicin in tumor but not in normal tissue. If these data could be verified in humans, it may have relevance to cancer chemotherapy.



	R1	R2
Epicatechin	H	H
Epicatechin-3-gallate	Gallate	H
Epigallocatechin	H	OH
Epigallocatechin-3-gallate	Gallate	OH

1.4.2.1.2 Tea Antioxidants and Free Radical Scavenging Activity

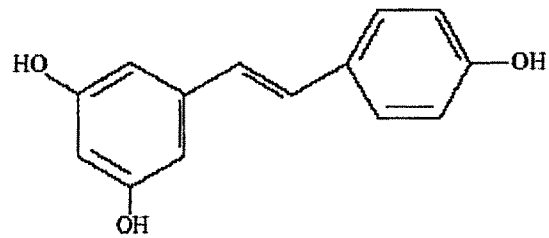
The generation of ROS in biological systems, either by normal metabolic pathways or as a consequence of exposure to chemical carcinogens, contributes to the multistage process of carcinogenesis (Agarwal and Mukhtar, 1993). Several reports suggest that peroxides and superoxide anion produce cytotoxicity and genotoxicity in the cellular systems (Perchellet and Perchellet, 1989). The source of H_2O_2 in cells and tissues is mainly through SOD-mediated dismutation of xO_2, which is generated in the cells and tissues by endogenous enzyme systems as well as by the nonenzymatic pathways (Perchellet and Perchellet, 1989). In addition, the highly reactive hydroxy radical (xOH) generated from H_2O_2 is known to damage DNA to produce pathological alterations. Cancer chemoprevention studies have shown that the levels of antioxidant enzymes are elevated in various organs of the test animals following administration of chemopreventive agents (Laskin et al., 1992). Compared to that observed in non green tea polyphenol (GTP) fed control group of mice, the oral feeding of GTP in mouse drinking water significantly increased glutathione peroxidase, catalase, and quinone reductase (QR) activities in small bowel, liver, and lungs (Khan et al., 1992). Extensive experimental data show that anticarcinogenic properties of tea are due to multifactorial antioxidative effects of epicatechin derivatives (ECDs, polyphenols) present therein.

Katiyar et al. (1994) found that EGCG, EGC, and ECG from green tea significantly inhibit Fe31 or ADP-supported spontaneous lipid peroxidation in mouse epidermal microsomes. Each of these ECDs was also effective in inhibiting photo enhanced LPO generated by incubating epidermal microsomes in the presence of photosensitizer, silicon phthalocyanine, and at 650 nm irradiation. At equimolar basis, EGCG, which is also the major constituent in GTP, showed maximum inhibitory effects compared to other ECDs. This study provides evidence for the antioxidative property of ECDs. Terao et al. (1994) also showed the antioxidative property of EC and ECG by measuring the inhibition of lipid peroxidation in large unilamellar liposomes composed of egg yolk phosphatidylcholine. This study provided the evidence that EC and ECG serve as powerful antioxidants against lipid peroxidation when phospholipid bilayers are exposed to aqueous oxygen radicals (Terao et al., 1994). Oral administration of green tea inhibited the formation of 8-

hydroxydeoxyguanosine in mice (Xu et al., 1992), and topically treated GTP inhibited hydrogen peroxide formation (Laskin et al., 1992). Tea polyphenols may inhibit carcinogenesis through their antioxidative activities supported by finding that catechin inhibited NNK-induced DNA single-strand breaks in rat hepatocytes (Yang and Wang, 1993). Recently, Miller et al. (1996) reported the antioxidative properties of black tea polyphenols by investigating their abilities to scavenge free radicals in the aqueous and lipophilic phases. Tea polyphenols can also react with peroxy radicals and thus terminate lipid peroxidation chain reactions. Reactive oxygen species play important roles in carcinogenesis by damaging DNA, altering gene expression, or affecting cell growth and differentiation (Cerruti, 1989).

1.4.2.2 Resveratrol

Resveratrol (trans-3, 4', 5-trihydroxystibene) occurs naturally in grapes and a variety of medicinal plants. In plants, resveratrol functions as a phytoalexin that protects against fungal infection (Hain et al., 1990). Because of its high concentration in grape skin, significant amounts of resveratrol are present in wine. *In vivo*, *ex vivo* and animal experiments have shown that resveratrol possesses many biological attributes that favor protection against atherosclerosis, including antioxidant activity, modulation of hepatic apolipoprotein and lipid synthesis, inhibition of platelet aggregation and the production of pro-atherogenic eicosanoids by human platelets and nutrophils (Soleas et al., 1997). It also has been reported to have cancer chemopreventive activity (Jang et al., 1997). This review focuses on the effect of resveratrol on chemoprevention of cancer and cardiovascular disease.



1.4.2.2.1 Chemoprevention of Cancer by Resveratrol

A newer dimension in the management of neoplasia is the increasing awareness that chemoprevention, which refers to the administration of chemical agents to prevent the initiational and promotional events associated with carcinogenesis, may be the most direct way to reduce mortality and morbidity of cancer. The process of chemical carcinogenesis can be divided into three general stages, and chemopreventive agents have been categorized according to the stages that they inhibit (Wattenberg, 1993). Resveratrol is an antioxidant, and it may suppress tumor development through the removal of ROS. A recent report shows that resveratrol is a potent cancer chemopreventive agent in assays representing three major stages of carcinogenesis, and the ability to inhibit cellular events associated with tumor initiation, promotion and progression has been attributed to the anticycloxygenase activity cyclooxygenase- 1, (Cox-1) of resveratrol. It has been found to induce phase II drug-metabolizing enzymes and to induce human promyelocytic leukemia cell differentiation. In addition, it has been found to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary gland in culture and to inhibit tumorigenesis in a mouse skin cancer model (Jang et al., 1997).

Resveratrol was found to act as a potential inhibitor of inducible NO synthase (iNOS) and inducible Cox-2 (Subbaramaiah et al., 1998; Tsai et al., 1999). Since physiological activity of iNOS and Cox-2 may benefit the organism, aberrant or excess expression of either iNOS or Cox-2 has been implicated in the pathogenesis of many disease processes, such as carcinogenesis and cardiomyopathy (Chhatwal et al., 1994; Dubois et al., 1996; Takahashi et al., 1997). Because iNOS and Cox-2 are essential components of the inflammatory response, which can ultimate repair of injury and carcinogenesis (Moncada et al., 1991; Anggard, 1994; Nathan and Xie, 1994; Seibert and Masferrer, 1994; Salvemini et al., 1994; Tamir and Tannenbaum, 1996). Strong antiproliferative properties of resveratrol have been found, and they are likely to be due to its ability to efficiently scavenge the essential tyrosyl radical of the small protein of ribonucleotide reductase and, consequently, to inhibit deoxyribonucleotide synthesis (Fontecave et al., 1998). The ribonucleotide reductase provides proliferating cells with

deoxyribonucleotides required for DNA synthesis during the early S-phase of the cell cycle (Reichard et al., 1987).

Another report shows that resveratrol may have therapeutic potential against liver injury through regulation of functions of hepatic stellate cells and kupffer cells (Kawada et al., 1998). In addition, Clément et al. (1998) have reported on a fascinating new facet of resveratrol. Using several experimental approaches, Clement et al. (1998) found that resveratrol acts as a cancer chemopreventive as well as a chemotherapeutic agent in humans. It has been found to induce apoptotic cell death in HL 60 leukemia cells as well as in T47D breast carcinoma cells at doses minimally toxic to normal peripheral blood lymphocytes.

1.4.2.2.2 Chemoprevention of Cardiovascular Disease by Resveratrol

Resveratrol has been proposed to explain, at least in part, the apparent ability of moderate consumption of red wine to reduce the risk of cardiovascular disease (Frankel et al., 1993a, 1993b; Bertelli et al., 1995; Pace-Asciak et al., 1995). It has been suggested that it plays a role in the prevention of heart disease, as it inhibits platelet aggregation, alters eicosanoid synthesis and modulates lipid and lipoprotein metabolism (Soleas et al., 1997). The role of platelets in initiating chemical signals that set in motion complex cellular events, resulting in atherosclerosis, following their adherence to the endothelial surface of arteries, as well as in triggering lumenal occlusion, leading to acute coronary heart disease (CHD), is well established (Renaud et al., 1992; Elwood et al., 1991). The potential of platelets to adhere to vascular endothelium as well as to participate in blood coagulation and thrombus formation can be measured based on their ability to aggregate *in vitro* in response to a number of agonists. Using ADP and thrombin as agonists, Pace-Asciak et al. (1995) demonstrated a dose dependent inhibition by resveratrol of the aggregation of platelets prepared from healthy human subjects.

Resveratrol was found to act as effectively as estradiol in stimulating progestrone receptor gene expression in MCF-7 breast carcinoma cells, but no superagonistic effect was found. In addition, it inhibited binding of 125I-labeled estradiol to estrogen receptor (ER) in competition binding studies, using nuclear extracts of MCF-7 cells (Frankel et al., 1994; Bertelli et al.,

1995). On the other hand, in comparison with MCF-7 cells, the superagonistic effect of resveratrol was less pronounced in BG-1 ovarian carcinoma cells, suggesting a partial tissue specificity (Jang et al., 1997). Although the concentration of resveratrol with estrogenic properties may have undesirable side effect, it can result in the stimulation of human breast cancer cells. This study raises the interesting possibility that this phytoestrogen may contribute to the cardioprotective effects associated with red wine consumption.

1.4.2.3 Melatonin

The discovery of melatonin as a direct free radical scavenger and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes has greatly increased interest in the use of this agent in the experimental and clinical setting (Tan et al., 1993). Its potential utility in humans is supported by its very low toxicity, its availability in a pure form and the fact that it is inexpensive (Reiter, 1998). Beyond its antioxidant activities, melatonin has been tested for and successfully used in other clinical situations. Perhaps it was initially taken by transmeridian travelers to quell the severity of jet lag. Thereafter, it became popular as a sleep promoting agent and interest in its use in the suppression of growth of certain cancer types is supported by experimental and clinical observations of a number of scientists (Cardinali, 2004; Lissoni et al., 2001). More recently, melatonin has been used as an adjunct treatment in newborn infants suffering with gram-negative bacterial infections and respiratory distress syndrome (Gitto et al., 2004). Both of these serious conditions are believed to be linked to massive toxic free radical generation and the associated tissue damage (Gitto et al., 2002).

The molecular damage that is caused by toxic reactants is often referred to as oxidative stress and the accumulation of these functionally impaired molecules contributes to physiological deterioration and disease development. Additionally, the free radical theory of aging espouses that the accumulation of injured, essential molecules also accounts for tissue deterioration during aging (Harman, 1999).

1.4.2.3.1 Oxidative Stress and Melatonin As An Antioxidant

Melatonin is both a direct free radical scavenger and an indirect antioxidant because of its ability to promote the activities of a variety of antioxidative enzymes (Rodriquez et al., 2004). While the direct free radical scavenging actions of melatonin are receptor independent, the indirect antioxidative functions may well be mediated by receptors, either located in the membranes of cells or intracellularly. Besides melatonin, however, there are a large number of other molecules that function as efficient antioxidants, the best known of which include vitamin E vitamin C and ß-carotene. Preliminary evidence suggests that melatonin works synergistically with these important antioxidative agents (Gitto et al., 2001).

The efficiency of an antioxidant to neutralize a toxic reactant depends on several factors. Besides the ease with which it donates an electron, its distribution within the cell is equally important. The most reactive reactants (e.g. •OH) travel only a few molecular diameters before they interact with another bystander molecule. Thus, for an antioxidant to prevent this damage it must essentially be at the site where the •OH is generated. Clearly then, the unique solubility of an antioxidant determines its efficacy. As a consequence, lipid soluble antioxidants such as vitamin E are particularly effective in directly detoxifying radicals in the lipid-rich environments of the cell, i.e. cellular membranes. Vitamin E is, however, less effective in protecting nuclear DNA from oxidative mutilation because of the aqueous environment in the nucleus. Conversely, vitamin C, which is readily water soluble, is highly beneficial in the aqueous regions of the cell, particularly the cytosol, and less so in membranes. In this regard, melatonin seems to be somewhat unusual. While it is clearly a lipid soluble agent, it seems also capable of entering the aqueous environments of the cell. This apparently amphiphilic nature allows melatonin to be protective of membranes, cytosolic molecules and nuclear DNA from free radical damage (Reiter et al., 2002). As with other antioxidants, what has been difficult to determine is what portion of the protection melatonin provides is a consequence of its direct free radical scavenging ability and what percentage is due to other actions of the indole, e.g. stimulating antioxidative enzymes (Rodriquez et al., 2004), or reducing electron leakage at the mitochondrial level, thereby reducing free radical generation (Acuna-Castroviejo et al., 2001).

Another feature that possibly increases the efficacy of melatonin in reducing oxidative stress is that the metabolites which are produced during the scavenging actions of melatonin, i.e. cyclic 3-hydroxymelatonin (cyclic 3-OHM), N-acetyl-N"-formyl-5- methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMF) seem also to be efficient scavengers (Tan et al., 2002). Thus, the second and third generations metabolites of melatonin may well contribute to the ability of the parent molecule to protect against oxidative stress. Because of this, rather than scavenging a single radical, melatonin *via* an antioxidant cascade may neutralize a number of toxic reactants (Tan et al., 2002). While such measurements are difficult to make, theoretically at least these assumptions are supported by available data. Comparisons of the relative efficacies of melatonin with classic antioxidants in protecting against oxidative damage have been carried out. Less so under *in vitro* conditions but especially *in vivo*, melatonin has consistently proven to be more effective than vitamins E and C in reducing molecular damage that occurs under high free radical generating conditions (Lopez-Burillo et al., 2003). A proposed obstacle to melatonin being a relevant physiological antioxidant is its reported low concentration within organisms or within cells. It is often assumed that melatonin is in equilibrium within organisms and the basis of reference for its concentration is its level in blood. Even at night when maximal secretion of melatonin from the pineal gland occurs, blood concentrations are in the low nanomolar range. This value is very much lower than the levels of other antioxidants such as vitamin C or the intracellular antioxidant, reduced glutathione. However, it is now apparent that melatonin levels in other bodily fluids or tissues may in fact be orders of magnitude higher than blood concentrations. This became readily apparent when melatonin was measured in fluids such as the bile and cerebrospinal fluid of the third ventricle (Skinner et al., 1999). Likewise, it has been preliminarily estimated that mitochondria, a major site of free radical generation, also contain much higher concentrations of melatonin than exist in the serum at the same time (Martin et al., 2000). Furthermore, besides the pineal gland, a variety of other cells have the capacity to synthesize melatonin, e.g. photoreceptor cells of the retinas and enterochromaffin cells in the gut. In these specific cells and in the adjacent areas, the concentrations of melatonin are again much higher than levels found in the

blood. When these cells generate melatonin it may be used within these cells or as an autocrine or paracrine secretion. It is well established that the levels of melatonin in the blood are primarily of pineal gland origin or, under some limited conditions, derived from the gastrointestinal tract (Huether, 1993). It has been shown that even the amounts of melatonin secreted by the pineal gland contribute to free radical protection. Thus, removal of this source of melatonin by surgical pinealectomy has been shown repeatedly to exaggerate the amount of molecular destruction resulting from high free radical states (Kilic et al., 2002).

Melatonin's high efficacy in reducing oxidative damage may involve both receptors independent as well as receptor-mediated processes. In addition to direct free radical scavenging, antioxidative functions of melatonin may include synergistic actions with classic antioxidants, stimulation of the synthesis of the important intracellular antioxidant GSH, its unique intracellular distribution, the fact that second and third generation metabolites of melatonin are also effective scavengers, its ability to induce gene expression and activities of antioxidative enzymes, its ability to reduce free radical generation at the mitochondrial level , as well as yet undefined actions (Rodriquez et al., 2004; Reiter et al., 2000). It is clear that while melatonin is protective against oxidative stress, the mechanisms whereby it achieves this high level of protection requires more extensive investigation.

1.4.2.4 Lovastatin

Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase that has been used in the clinic to treat hypercholesterolemia (Downs et al., 1998)). Lovastatin has also been shown to arrest tumor and normal cells in the G1 phase of the cell cycle and has demonstrated antitumor effects in experimental murine models (Rao et al., 1998; Matar et al., 1999). Although clinical studies on the antitumor activity of lovastatin have been initiated (Thibault et al., 1996), its effectiveness in clinical tumor therapy will probably depend on whether combination therapies are found in which lovastatin used with other drugs is shown to exert potentiated antitumor effects. As shown recently by Agarwal et al. (1999), pretreatment with lovastatin increased apoptosis induced by chemotherapeutic agents in

tumor cells in vitro. Lovastatin has also been shown to strengthen the antitumor activity of cisplatin and tumor necrosis factor ∝ in murine tumor models (Feleszko et al., 1998; Feleszko et al., 1999).

HMG CoA reductase inhibitors (statins) promote reduction in plasma levels of low-density lipoprotein (LDL) cholesterol, a primary risk factor in coronary artery disease. Numerous primary and secondary prevention trials confirm clinical benefits with this class of agents (Jones et al., 2003). The mechanisms involved have largely been attributed to the ability of these agents to inhibit cholesterol biosynthesis, leading to upregulation of hepatic LDL receptors and corresponding reductions in circulating levels of low-density lipoprotein (LDL) and very high density lipoprotein particles by increasing catabolism. Additionally, a significant increase in high-density lipoprotein (HDL) is produced which ultimately results in favorable lipid ratio (Rader et al., 2003). All these lipid actions have been strongly suggested to result in higher percentage of patients achieving National Cholesterol Education Program (US Department of Health and Human Services) and European LDL cholesterol goals [Shepherd et al., 2003), However, a growing body of evidence suggests that some of the clinical benefits of statin therapy may be attributed to mechanisms independent of their cholesterol-lowering effects (Xu et al., 2004; Tanaka et al., 2001). These so called *pleiotropic effects* are defined as, producing or having multiple effects from a single gene. Such effects are believed to include antiinflammatory actions, property to reverse endothelial dysfunction by prevention of LDL oxidation and increasing nitric oxide bioavailability. Their antioxidant actions and ability to provide plaque stability, favorable coagulation profile, preventing platelet aggregation and normalizing sympathetic outflow as well as their antiproliferative and immunosuppressive properties suggest a new face of statin therapy which make them very important not only in the treatment of dyslipidemias and associated complications but also in many other disease states.

1.4.2.4.1 Statins and endothelial dysfunction

Endothelial dysfunction has relevance to the pathogenesis, progression and prognosis of a wide spectrum of cardiovascular diseases. It is characterized by reduced bioavailability of NO, LDL-oxidation in the

vascular wall and the vascular inflammatory response. All these pathological processes fundamental to the development and progression of endothelial dysfunction are modulated by increased vascular oxidative stress in dyslipidemia (Fenster et al., 2003). Statins have been shown to improve endothelial dysfunction by increasing nitric oxide bioavailability as well as by reducing LDL oxidation and vascular inflammatory response.

a) Effect of statin therapy on nitric oxide bioavailability

Nitric oxide is a crucial mediator of cardiovascular homeostasis. In vascular endothelium, statins increase the concentration of nitric oxide, which has vasodilator, antithrombotic and antiproliferative properties (Takemoto et al., 2001). Recent cell culture studies demonstrate that statin therapy suppresses superoxide formation and enhances NO generation by vascular endothelial cells *via* inhibition of isoprenylation of Rac and Rho (Wassmann et al., 2002). Rac is a component of the NAD(P)H oxidase complex of both leukocytes and vascular cells whereas Rho is a small GTPase involved in cell signaling (Laufs et al.,1998). Inhibition in Rho isoprenylation in endothelial cell has been shown to mainly result in enhanced NO production. Because Ras and Rho also regulate the cell cycle, they are, in addition, likely targets for the direct antiproliferative effects of statins. Indeed, statins inhibit vascular smooth muscle cell proliferation in transplant-associated arteriosclerosis (Kobashigawa et al., 1995). While others have shown that statins enhance the activity of eNOS through protein kinase activation (Kureishi et al., 2001). Simvastatin has been shown to enhance the production of NO in the vascular endothelium and attenuate myocardial injury following ischemia and reperfusion in normocholesterolemic, hypercholesterolemic mice (Scalia et al., 2001). A recently introduced new statin (rosuvastatin) has been shown to increase vascular endothelial NO production and attenuate myocardial necrosis following ischemia and reperfusion in mice, thereby providing cardio-protective effects independent of lipid-lowering actions (Pelat et al.,2003).

b) LDL oxidation

Oxidation of LDL cholesterol is critical to the pathogenesis of endothelial dysfunction. Oxidative modification of LDL appears to play a key role in mediating the uptake of lipoprotein cholesterol by macrophages and

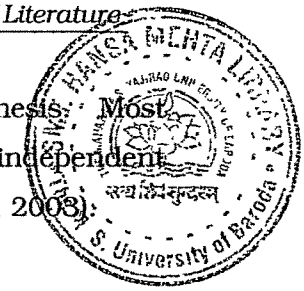
in other processes including cytotoxicity within lesions. LDL cholesterol is subsequently oxidized by superoxide generated by macrophage NAD(P)H oxidase. Hypercholesterolemia potentiates LDL oxidation by increasing substrate and promoting LDL conformations that are more susceptible to oxidation. Oxidized LDL mediates a number of redox-sensitive processes that are deleterious to endothelial function. Through inhibition of eNOS and inactivation of NO, oxidized LDL promotes an inflammatory phenotype through activation of NF- B triggering an elaboration of inflammatory cytokines and adhesion molecules through redox sensitive pathways. Inflammatory mediated release may in turn activate enzymatic source of reactive oxygen species, including NAD(P)H oxidase and xanthine oxidase, potentiating the already established oxidative stress. Thus, this can lead to a self-perpetuating cycle (Fenster et al., 2003). Statins reduce the susceptibility of lipoproteins to oxidation both *in vitro* and *ex vivo* i.e. they decrease the LDL oxidation (Giroux et al., 1993). This is done by increasing NO which can scavenge superoxide free radical anions responsible for LDL oxidation, by inhibiting NAD(P)H oxidase, the inflammatory cascade or through their antioxidant actions.

c) Vascular inflammatory response

Vascular inflammation is also supposed to account for endothelial dysfunction. Statins by their vascular antiinflammatory actions can improve endothelial dysfunction.

1.4.2.4.2 Antioxidant actions of statin therapy

Reactive oxygen species including superoxide ($O_2^{\bullet-}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO·) have oxidative property and contribute to oxidative stress. Normal endothelial function is characterized by a dynamic balance between NO and other oxidants. As a scavenger of superoxide anions, the potent vasodilator and antioxidant NO antagonizes the vasoconstrictive properties of the ROS. Thus, statins possess antioxidant properties by increasing the NO bioavailability, by reducing lipid peroxidation and ROS production (Wilson et al., 2001; Wassmann et al., 2001). One recent study suggested that statins promote systemic antioxidant effects through the suppression of distinct oxidation pathways. The major pathways inhibited include myeloperoxidase-derived



and nitric oxide-derived oxidants, implicated in atherogenesis. Most importantly, this study suggested that these effects were largely independent of lipid-lowering and antiinflammatory actions (Shishehbor et al., 2003).

1.4.2.4.3 Cancers

Lovastatin not only induces apoptosis, but also promotes redifferentiation in anaplastic thyroid cancer cells, and this suggests that the statins merit further investigation as differentiation therapy for the treatment of anaplastic thyroid cancer (Wang et al., 2003). One study identified a subset of various pediatric cancers and squamous cell carcinomas that are sensitive to lovastatin-induced apoptosis and this study showed HMG-CoA reductase as a potential therapeutic target of these cancers (Dimitroulakos et al., 2001). Because Ras and Rho also regulate the cell cycle, they are, in addition, likely targets for the direct antiproliferative effects of statins. Indeed, statins may have clinical benefits in inhibiting certain breast cancers (Denoyelle et al., 2001).