Chapter 2

REVIEW OF LITERATURE

2.1 Drugs & Medicine

Advancements along with developments in chemistry, pharmacology, genetics, molecular biology and lab technology led to modern medicine. A drug cannot be defined by a single definition as it has different meaning in drug control laws, government regulations, medicine and colloquial usage. Thus a drug can be broadly defined as any substance when absorbed into the body of living organisms alters bodily function. In pharmacology a drug is a chemical substance used in the treatment, cure, prevention or diagnosis of diseases or used to enhance physical and mental well being. The word drug is thought to originate from old French “drogue” possibly deriving later into “droge-vate” from middle Dutch meaning “dry barrels” referring to medicinal plants preserved in them.

A medicine is a drug taken to cure or ameliorates any symptoms of illness or medical condition. Medicine dispensing is often regulated by government into three categories: over the counter (OTC) which are available in pharmacies and supermarkets without restrictions; behind the counter (BTC) which are dispensed by pharmacist without needing a doctor’s prescription and Prescription only medicine (POM) which must be prescribed by a licensed medical professional usually a physician.

Some government define drug by laws. In United States the Federal Food, drug and cosmetic Act define drug as articles intended for the use in the diagnosis, cure, mitigation or prevention of diseases in man or other animal and articles intended to affect the structure of or any function of the body of man or other animals. Preclinical investigations on reproductive toxicology, mutagenicity and carcinogenicity are mandatory. Although a great deal is known about pharmacokinetic changes during development,
information regarding developmental changes in pharmacodynamics (medicine action and toxicity) is limited.

2.2 Marketed Pharmaceuticals, Genetic Toxicology & Drug Development

Genetic toxicology is the study of substances that induce DNA damage, the mechanisms of DNA damage, and the response to such damage by the test system (cell or animal). DNA damage can be identified and quantified as the frequency of DNA adducts, strand breaks, mutations, or chromosome aberrations. DNA damaging substances cannot be developed as pharmaceutical compounds except for some situations where unmet medical need, life-threatening diagnoses, or short-life expectancy exists. Increasingly, in an environment where multiple drugs exist to treat common ailments, even a single, isolated positive genetic toxicology finding can result in unfavorable product labeling and negatively impact marketability of the drug. Genotoxicity data in combination with acute and subchronic animal toxicity data is used by health regulators as a basis to approve Phase 1 clinical trials in healthy subjects or restrict trials to patient populations.

Because there is a strong desire to conduct Phase 1 clinical trials in healthy subjects to obtain information on metabolism, most pharmaceutical companies conduct screening versions of the genetic toxicology regulatory tests required to file an investigational new drug (IND) application. The International Committee on Harmonization (ICH) has developed guidelines defining the timing and the specific test battery required to file an investigational new drug (IND) application requesting approval to begin first-in-human (FIH) studies.

Genotoxic/carcinogenic potential has to be stated for numerous compounds which are often in pharmaceutical use. Independent and thorough testing is to be done for new compounds for which information is incomplete with respect to their interaction with macromolecules or that
have the potential to generate reactive metabolite in the body. *In vivo* cytogenetic assays (micronucleus/chromosome aberrations/sister chromatid exchanges) are not made essential requisite for the regulatory bodies which is substituted by the equivalent *in vitro* assays. Consequently, adequate *in vivo* cytogenetic data are not available for many of the pharmaceuticals. However, the *in vivo* test is especially relevant to assess genotoxicity hazard because it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes and is also useful in further investigation of a mutagenic effect detected by an *in vitro* genotoxicity test (Krishna and Hayashi, 2000).

Examination of sperm head morphology is one of the inexpensive and prerequisite tests of the functional capacity of semen in reproduction. Substances of demonstrated genotoxic activity can induce alterations in all types of cells. Thus, the characterization of their deleterious effects, both in somatic and germinal cells, can be highly informative, especially in cases of substances frequently used in general human therapy. Sperm are highly susceptible to reactive oxygen species (ROS) that can damage sperm DNA and structure, resulting in reduced fertilizing capacity. Exposure to radioactive contamination can also impair sperm swimming behavior and fertilizing ability, both through a reduction of sperm DNA integrity and via an increased generation of reactive oxygen species (ROS). Wyrobek, *et al.*, 1983 reported that large reductions in sperm number or motility or large increases in sperm with abnormal shapes are associated with reduced fertility. Since mutations in the germ line cells are the only cells capable of transferring a mutation to the next generation, therefore, more studies are required on the effect of pharmaceuticals in the germ cells.

The drugs selected for our study are metronidazole and artesunate. In developed western countries metronidazole and artesunate are prescribed drug. However in developing countries like India these drugs are widely available without prescription and are used indiscriminately. Thus it
necessitates the induction of multiple cytogenetic endpoint analysis to evaluate the genetic toxicity caused by the drugs.

2.3 Drug and Background Radiations

Radiation is a well-known mutagenic and carcinogenic agent. Naturally-occurring background radiation is the main source of exposure. People are unknowingly exposed to background radiation either naturally or man-made (diagnostic and therapeutic). Our lack of knowledge about the behaviour of substances in combination is often avoided by assuming that the toxicity of a mixture is simply the sum of the expected effects from each component, i.e. synergistic or antagonistic interactions (Ergene et al., 2007). Naturally-occurring background radiation is the main source of exposure. Ionizing radiations, from natural and man-made sources i.e. diagnostic and therapeutic devices (e.g. X rays, radiotherapy, and positron emission), interact with biological tissue and can generate a trail of structural and chemical modifications of the DNA helix. It is well established that ionizing radiation causes single strand breaks, double strand breaks; oxidative damage, chromosomal aberration and mutation lead to the apoptosis through generation of toxic free radicals (Sankaranarayanan, 2006). Except for patients who are treated with both chemical and physical agents, medical employees such as nurses, pharmacy personnel and staff working in radiological units might be exposed to low doses of radiation and drugs. The effort to predict the genetic consequences for humans of exposure to ionizing radiation has certainly been one of the most important issues of human genetics in the past 50 years (UNSCEAR, 2001). Occupational exposure to ionizing radiation may result in DNA damage leading to chromosome aberrations (Kubelka, et al., 1992; Pohl-Ruling, 1992; Garaj-Vrhovac V. et al., 1997). The importance of chromosome aberrations for evolution and their association with human health have been recognized for almost a century. A fraction of chromosome aberrations induced in germ cells are transmitted to the next generation.
and this is of great concern from the point of view of genetic risk. Given that the majority of chromosome aberrations and many gene mutations lead to inherited diseases, the analysis of radiation-induced changes in germ line mutation rates could provide important data on the genetic risk of human exposure to ionizing radiation. Because ionizing radiation with a low linear energy transfer (LET) can generate hydroxyl radicals and it is possible to perform uniform irradiation of relatively large samples such as the whole body of a mouse, gamma- or X-rays would be suitable as a possible source of hydroxyl radical generation, which is a major cause of DNA damage \textit{in vitro} and \textit{in vivo} (Kiefer, 1990). The effects of low LET (linear energy transfer) radiations are caused mainly by the generation of reactive oxygen species (ROS). These ROS interact with biological molecules producing toxic free radicals leading to lipid peroxidation and DNA damage (Jagetia, \textit{et al.}, 2005).

2.4 Reactive oxygen species, Oxidative Stress and Antioxidant Defense

A free radical is an atom or group of atoms that have one or more unpaired electrons. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can create havoc on a broad range of macromolecules. A prominent feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells. There are many types of radicals, but those of most concern in biological systems are derived from oxygen, and known collectively as reactive oxygen species. Oxygen has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. In the sequential univalent process by which \textit{O}_2 undergoes reduction, several reactive intermediates are formed, such as superoxide (\textit{O}_2^-), hydrogen peroxide
(H$_2$O$_2$), and the extremely reactive hydroxyl radical (.OH): collectively termed as the reactive oxygen species (ROS).

Under the conditions of normal metabolism the most important source of O$_2$\textsuperscript{-} is the mitochondrial electron transport chain, which leaks a few electrons directly onto O$_2$ as part of normal metabolism. It is estimated that 1\% to 3\% of O$_2$ reduced in mitochondria is in the form of O$_2$\textsuperscript{-} (Turrens, 2003). This comes from two sites, complex 1 (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase), with the latter being the major source under normal conditions (Finkel, et al., 2000). Several enzymes also contribute to O$_2$\textsuperscript{-} production. One of the best characterized is xanthine oxidase, which is present in the cytosol of many tissues but also can be found in circulating blood and bound to glycosaminoglycan sites in the arterial wall (White, et al., 1996). Normally the enzyme acts as a dehydrogenase and transfers electrons to NAD\textsuperscript{+} rather than O$_2$, but in ischemia reperfusion (Ullrich, et al., 2000; Mueller et al., 2005) or in sepsis (Mueller et al., 2005; Brandes, et al., 1999) the active site of the enzyme is oxidized and the enzyme acts as an oxidase and produces O$_2$\textsuperscript{2-}. Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage.

Many antioxidants work by transiently becoming radicals themselves. These molecules are usually part of a larger network of cooperating antioxidants that end up regenerating the original antioxidant. For example, vitamin E becomes a radical, but is regenerated through the activity of the antioxidants vitamin C and glutathione.

**Enzymatic Antioxidants**

Three groups of enzymes play significant roles in protecting cells from oxidant stress:
Superoxide dismutases (SOD) are enzymes that catalyze the conversion of two superoxides into hydrogen peroxide and oxygen. The benefit here is that hydrogen peroxide is substantially less toxic that superoxide. SOD accelerates this detoxifying reaction roughly 10,000-fold over the non-catalyzed reaction.

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O_2^- + O_2^- \xrightarrow{\text{SOD}} O_2 + H_2O_2
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SODs are metal-containing enzymes that depend on bound manganese, copper or zinc for their antioxidant activity. In mammals, the manganese-containing enzyme is most abundant in mitochondria, while the zinc or copper forms predominant in cytoplasm. Interestingly, SODs are inducible enzymes - exposure of bacteria or vertebrate cells to higher concentrations of oxygen results in rapid increases in the concentration of SOD. Catalase is found in peroxisomes in eucaryotic cells. It degrades hydrogen peroxide to water and oxygen, and hence finishes the detoxification reaction started by SOD. Glutathione peroxidase is a group of enzymes, the most abundant of which contain selenium. These enzymes, like catalase, degrade hydrogen peroxide. They also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants.

**Non-enzymatic Antioxidants**

The non-enzymatic antioxidants of particular importance are:

Vitamin E is the major lipid-soluble antioxidant, and plays a vital role in protecting membranes from oxidative damage. Its primary activity is to trap peroxy radicals in cellular membranes.

Glutathione may well be the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (glutamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphhydryl group (SH) that is very reactive, providing an abundant target for radical attack.
The dietary intake of antioxidants is thought to play a major role in enhancing defense against ROS. The term antioxidant is broadly defined as any substance that prevents the oxidation of biomolecules either directly by scavenging reactive oxygen species or indirectly by upregulating the antioxidant defense or repair system. Antioxidants like vitamin C, Vitamin E, Carotenoids and some flavonoids have been identified in many food products. Natural products also contain mixtures of other antioxidants and other bioactive compounds with unknown antioxidant properties. It has been revealed that dietary antioxidants, decreases the risk of oxidative stress. In this context, the need for effective antioxidants aimed at minimizing the oxidative stress and providing defense against free radical induced damage in diverse clinical and pathological conditions has gained significant importance (Priyadarsini, 2005). Investigations for effective and nontoxic compounds with radioprotection capability led to increasing interest in naturally occurring dietary antioxidant such as curcumin and vitamin C etc.

Vitamin C is a vital antioxidant that acts as a free radical scavenger and may regenerate other antioxidants, including vitamin E and help in protecting these kind of damaged cells (Chan, 1993). It was established that the distribution of ascorbic acid and its concentration in the organs was subjected to variations depending on introduction of different drugs (Linster, 2007). The role of vitamin C in protecting against oxidative DNA damage is a matter of much controversy (Rivière, et al., 2006). On one hand, numerous studies point to a protective effect of vitamin C supplements (Ajey, et al., 1992, Sardas, et al., 2006; Demirba et al., 2006, Harapanhalli, et al., 1996, Song, et al., 2006). On the other hand, several studies have suggested short-term effects, no effect, or even a prooxidant effect (Moertel, et al., 1985; Audera, et al., 2001).

Curcumin, an antioxidant derived from turmeric (Curcuma longa, Zingiberaceae) and known to possess therapeutic properties since ancient
2.5. Test Drugs

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2.5.1 Metronidazole

Metronidazole (MTZ, 1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole), an antiparasitic and antibacterial compound, is one of the world’s most used drugs. It is among the top 100 prescribed drugs in the US (Rxlist, 1999) and one of the 10 most used drug during pregnancy (Thapa, et al., 1998). In addition metronidazole is relatively inexpensive (Prim care update, 1996) thus making its consumption more frequent. It appears in the essential drug list of the world Health Organization (WHO, 1994). This drug has activity against protozoan’s like Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis and gram-negative anaerobes such as Helicobacter pylori, for which the drug has been proved an effective treatment (Freeman, et al., 1997). In addition MTZ has been applied as a radiosensitizer of hypoxic or tumor cells (Rauth, 1984). MTZ has been found to interact with and bind to DNA of both bacterial and mammalian cells under anaerobic conditions (La Russo et al., 1977, Probst, et al., 1983; Sina, et al., 1983).
An increase in the mutagenicity of serum in female mice 8 hr after administration of metronidazole was observed (Ings, et al., 1974). Furthermore, it has been observed that metronidazole induces DNA single strand breaks in the lymphocytes of the patients on standard doses of the drug (Reitz, et al., 1991). In the comet assay, the metronidazole treatment leads to an increase in tail length, which is at par with other in vitro investigations on MTZ, induced DNA damage (Gisell, et al., 2002; Re, et al., 1997; Menéndez et al., 2001). It has been observed that Studies reported negative result in dominant lethal test in rat and mouse (Wyrobek,1983) and negative results in the micronucleus test in mice (Bruce,1974). However, experimental data on genotoxic effects of metronidazole showed a linear dose-response increase in micronuclei in mice bone marrow cells in human lymphocytes cultures blocked with Cytochalasin B (Schmid,1973) A significant increase in chromosomal aberration in human lymphocytes cultures and abnormal anaphase in CHO (Chinese hamster ovary) cells were observed (Schmid,1973). On the other hand the drug can induce alterations in somatic and germinal cells (Grover, et al., 2001). It has been reported that after 6 weeks of treatment of rats with MTZ (400 mg/kg/day), decreases testicular weight, testicular and epididymal spermatozoon counts and causes abnormal spermatozoon morphology with degeneration of seminiferous tubules (Grover, et al., 2001). Abnormalities in the flagellum and the head as well as decrease in the number of motile spermatozoon have been reported in the morphological analysis after MTZ treatment (Nahas and Ashmawy, 2004; Mudry, et al., 1994). Depleted serum testosterone level in Metronidazole-treated Mice (Karbalay-Doust and Noorafshan, 2011) has also been reported.

### 2.5.2 Artesunate

Malaria is still one of the most deadly diseases in the developing countries. Malaria continues to be major global killer despite the continuous
effort to reduce its toll. Each year at least a million people die, pregnant woman and children below five years of age account for the majority. (WHO UNICEF, 2005). Artemisinin, a natural sequiterpene lactone peroxide of the qinhao plant (Artemisia annua). The earliest report of use of this plant was in Chinese prescription for 52 kinds of diseases dating from 168 B.C. for the treatment of hemorrhoids. The active component Artemisinin was extracted in 1970. Since than there are more than 1 million dosage of Artemisinin and its derivative have been used for the treatment of Malaria. With the evolution of drug resistance to malaria parasites, artemisinin antimalrials have become the first choice medication in several countries.

Artemisinin and its derivative such as artesunate, arteether, artemether, and dihydroartemisinin are the most potent antimalarial drugs available throughout the world. (Hein, et al., 1993). The Artemisinin derivative act rapidly on parasites leading to their quick elimination thereby rendering these derivatives effective against severe malaria. Artemisinin are the only antimalrials apart from the cinchona alkaloid used to treat severe malaria. Furthermore, Artemisinin has shown several advantages over quinine, including lack of stimulation of insulin and attended risk of hypoglycemia. (Nealon, et al, 2002, Hein, et al., 1993).

Among the various artemisinin derivative artesunate, water-soluble half esters succinate derivative has been the most commonly used derivative in the past 15 years, most clinicians feel that parenteral administration of the artesunate is the most effective treatment for severe malaria. (Nontprasert, et al., 1998, Asley, et al., 2005). Artesunate being the most commonly used derivative. Artesunate is available in oral, rectal and parenteral formulation providing rapid clinical effect in patients. The mechanism of action has not been fully elucidated. The structural determinant of the activity of artemisinins is the endoperoxide Bridge which is a specific feature of this type of compounds. It has been suggested that
the parasiticidal activity starts with the reaction of artemisinins with haem iron, leading to the generation of activated oxygen species, such as oxygen radicals, or of a C-centred radical of artemisinin itself, which are then further producing lethal damage to the parasite (Meshnick, et al., 1993; Olliaro, et al., 2001). Artesunate has been found to kill relatively young parasites when compared with Quinine or Chloroquine (Kuile, et al., 1993). Artesinin derivatives are fast acting substances, leading to a rapid clearance of the malaria parasites from the blood, while the short biological half-life precludes a long-lasting activity. Therefore, artemisinins are preferably used not in monotherapy, but in combination with longer-acting drugs that have a slower onset of activity. The WHO Roll Back Malaria programme has advocated such a strategy, recommending the use of artemisinin-containing therapies (ACT) in areas of emerging, high resistance to the most commonly used antimalarials. Malarial distribution is uneven across the Indian subcontinent; it is the major cause of infection in North east, Orissa and tribal settlements across the country. Moreover the first incidence of chloroquine resistance in India was reported in Assam in 1973. Thus, making the use of antimalarial drug other than quinine more frequent.