Chapter I
Introduction and Review of Literature
1. **Introduction**

Cancer has been described for the first time by the Egyptians (around 3000 BC) on the basis of its symptoms, but the term Cancer was pioneered by Hippocrates (460-370 BC) and is considered as the “Father of Medicine”. He used the terms carcinos and carcinoma for non-ulcer forming and ulcer forming tumors. In Greek, carcinos and carcinoma refers to crab, because of the symptoms around the tumor is like the finger like projections that resembles with the shape of a crab. Moreover, in ancient Egypt, there are evidences of bone tumors found among fossilized bones of human mummies.

Carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell occurs which lead to a progressively dysplastic cellular appearance, deregulated cell growth, and finally develops carcinoma. According to the estimates, in India, around 556,400 people died of cancer in 2010. (Dikshit et al., 2012) Cancer is a class of disease or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Cancer results from a multistage process that involves three distinguishable stages: initiation (Normal cell to transformed or initiated cell), promotion (initiated cell to preneoplastic cell), and progression (preneoplastic cell to neoplastic cell) (Brennan, 1975).

Hepatocarcinogenesis (Liver cancer), a process involved in cell transformation, is the fourth most common cause of cancer mortality in the world (Schutte et al., 2009), representing the 85% of liver cancers. Other types of liver cancer include cholangiocarcinoma and angiosarcoma (or haemangiosarcoma) which originates in the cells that line the bile duct and in the blood vessels of the liver respectively (Marra et al., 2011). Hepatitis viral infection, food additives, alcohol, aflatoxins, environmental and industrial toxic chemicals, cosmetics, food preservatives, air and water pollutants are the major risk factors of liver cancer (Ciemniak, 2006). With the advances in the scientific research, HCC is now decreasing in hepatitis B virus endemic countries due to the implementation of vaccination programs while it is increasing in cohorts who have been infected with chronic hepatitis virus C (Chokshi et al., 2001; Ohata et al., 2003).

The molecular mechanism underlying hepatocarcinogenesis is very complicated. Due to a progressive accumulation of mutations, cancer cells have defects in regulatory genes that govern normal cell proliferation and homeostasis. The alterations in cell physiology that collectively dictate malignant growth are: i) self-sufficiency in growth signals (activation of oncogenes); ii) insensitivity to growth-inhibitory signals (inactivation of anti-oncogenes or...
tumor suppressor genes); iii) escape from apoptosis; iv) limitless replicative potential; v) neo-angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Hepatocarcinoma is also widely considered to be a chemotherapy-resistant disease (Bruix et al., 2001). Sorafenib, the only drug approved by the United States Food and Drug Administration for the treatment of advanced HCC, increases the median survival time by less than 3 months (Llovet et al., 2008). However, this drug does not defer the symptomatic progression of the disease, costs about $5400 per month for treatment (Lu, 2010), and exhibits severe adverse effects, including a significant risk of bleeding (Je et al., 2009). These drawbacks dictate the exploration for novel preventive and therapeutic approaches for this dreadful disease. (Sporn and Suh 2000; Jordan, 2007)

Although there is no novel treatment, that can entirely surmount cancer, many types of the disease might be avoidable. Risk of cancer can be suppressed by eliminating the identified carcinogens or at least curtailing the exposure to them, but primary prevention is implemented only after the identification of the corresponding risk factors. Furthermore, the avoidance of some risk factors could require large lifestyle changes, which are not easy to implement.

Chemoprevention, a relatively new and promising strategy to prevent cancer, is defined as the use of natural dietary compounds and/or synthetic substances to block, inhibit, reverse, or retard the process of carcinogenesis. The chemopreventive effects elicited by these natural dietary compounds are believed to include anti-oxidative, anti-inflammatory activity, induction of phase II enzymes, apoptosis, and cell cycle arrest. Many mechanisms have been shown to account for the anti-carcinogenic actions of natural dietary compounds; attention has recently been focused on intracellular-signalling cascades as common molecular targets for various chemopreventive natural dietary compounds (Pan and Ho, 2008).

In the present scenario, there is a growing interest in the use of phytochemicals as chemopreventive agents against various types of cancers, due to their ability to selectively kill tumour cells and suppress carcinogenesis in preclinical animal models (Huang et al., 2010; Bishayee and Darvesh, 2010). Mounting evidence, based on in-vitro experiments and studies involving animal models as well as humans, support potential chemopreventive and therapeutic effects of diverse phytochemicals in liver cancer (Glauert et al., 2010; Bishayee et al., 2010). In the present research work, an attempt has been made to evaluate the hepatoprotective efficacy of various plants and phytochemicals against various
hepatotoxins and the role of these natural products in the chemoprevention of hepatocarcinogenesis.

2. Review of Literature

Cancer was described for the first time by Hippocrates as ‘karkinos’. Galeno introduced the word neoplasia only in the II century. He defined it as the growth of a body area adverse to nature (Gutiérrez and Salsamendi, 2001). Edwin Smith’s papyruses, dating from the XVII century, describe breast tumefaction. According to Hayes (1995), it was the English surgeon Percivall Pott who first recognized in 1775 the casual relationship between exposure to environmental substances and neoplastic development. This author described the occurrence of cancerous alterations in the skin of the scrotum of London chimney sweeps as a consequence of repeated localised contamination with soot. Some years later, and based on these observations, a guide distributed to Danish chimney sweeps recommended that these professionals take a daily bath to avoid such an occurrence (Hayes, 1995, Gutiérrez and Salsamendi, 2001). Still in the XVIII century John Hill observed a high proportion of nasal mucosa cancer in his patients, and traced it to the localised long-term exposure to snuff. In 1890, a high incidence of bladder cancer in chemical and rubber industry workers was observed across Europe. (Cohen and Ellwein 1991, Gomes-Carneiro et al., 1997; Garner, 1998; Dybdahl et al., 1999; Huff, 1999; Bertram 2001). By the end of the nineteenth century it had become evident that occupational exposure to certain chemicals or mixtures of chemicals had carcinogenic effects (Luch, 2005). The all-important next step was to systematically investigate and reproduce these diseases in experimental surroundings. The first experimental work on chemical carcinogenesis was carried out in 1915 by the pathologist Katsusaburo Yamagiwa and his assistant Koichi Ichikawa (Yamagiwa and Ichikawa, 1918). They rubbed rabbit ears with coal tar and observed the development of papillomas and carcinomas. Meanwhile, others researchers studied carcinogenesis of the bladder, liver, kidney, pancreas and lung using laboratory animals. Its success laid the foundations of the experimental use of animals in the study of human diseases (Toth, 2001). Later, Beremblum and Shubik used polycyclic aromatic hydrocarbons and croton oil to study skin carcinogenesis in mice and demonstrate that cancer development includes several stages (Beremblum and Shubik, 1947). When applied in low doses, none of these substances have carcinogenic properties by themselves. Yet, when mixed and in equal doses, they induced neoplastic development. The order of exposition to these substances was fundamental for carcinogenesis. Neoplasias developed only when the hydrocarbons were used first and then the croton oil,
never the other way around. These authors felt that the carcinogenic action of these substances was responsible for converting normal cells into neoplastic cells. For them, carcinogenesis was a complex process including one phase called initiation and another called promotion, with one or more genetic changes necessary for cancer development. During the next decade, Foulds (1954) introduced the term progression by studying breast adenocarcinoma in female mice. In the pre-Watson and Crick era, before carcinogens were known to bind to DNA, the cancers produced by chemical carcinogens were believed to be due to their interaction with proteins in specific tissues (Miller and Miller, 1952). By the end of the 1960s, increasing evidence pointed to a correlation between the DNA binding capacity of a particular carcinogen and its biological potency (Luch, 2005).

The factors responsible for cancer development are classified as exogenous and endogenous (Camargo et al., 1999, Gutiérrez and Salsamendi 2001). The first group includes nutritional habits (food preservation and preparation), socio-economic status, lifestyle, physical agents (ionising and non-ionising radiation), chemical compounds (natural and synthetic) and biological agents (Helicobacter pylori, Epstein Barr virus, human T lymphotropic viruses I and II, human papilloma virus and the hepatitis B virus, parasites such as Schistosoma haemotobium, Clonorchis sinensis and Opisthorchis vivarium; growth factors) (Pitot and Dragan, 1991, Barrett and Anderson, 1993, Farmer, 1994, Weisburger, 1999, Minamoto et al., 2000, Lutz, 2002). Unhealthy lifestyle habits such as: excess alcohol consumption; inhalation of tobacco and related products; the ingestion of certain foods and their contamination by mycotoxins; are responsible for higher incidences of certain types of neoplasias in a number of population groups (Gomes-Carneiro et al., 1997, Weisburger, 1999, Gutiérrez and Salsamendi, 2001). Endogenous factors include immune system damage and inflammation caused by uncertain aetiology (e.g. ulcerative colitis, pancreatitis, etc.), genetic makeup, age, endocrine balance and physiological condition (Cohen et al., 1991; Barrett and Anderson, 1993; Huff, 1994; Koivusalo et al., 1994; Weisburger, 1999; Minamoto et al., 2000; Gutiérrez and Salsamendi, 2001; Dewhirst et al., 2003; Ohshima et al., 2005).

Epidemiological studies of cancer incidence demonstrated that the risk of developing cancer varies between population groups and these differences are associated with lifestyle factors and habits (Garner, 1998; Lai and Shields, 1999; Gutiérrez and Salsamendi, 2001). Population migration has resulted in the development of types of cancer typical of particular geographical areas (King et al., 1995; Gutiérrez and Salsamendi, 2001). The relationship between chemical substances in the workplace and the development of certain
neoplasias in various occupational groups led to the conception of experimental models to better understand the bio-pathological processes inherent to carcinogenesis (Weinstein, 1991; Cohen et al., 1992; Gutiérrez and Salsamendi, 2001).

Boveri laid down the genetic basis of neoplastic development for the first time in 1914 with his theory of somatic mutation in cancer cells. However at the time, experts in the area of chemical carcinogenesis attributed little importance to this hypothesis, considering it to be pure speculation, instead choosing to put their faith in the lesser knowledge already available (Weisburger, 1999). Between 1980 and 1990, the discoveries made via the molecular biology of proto-oncogenes and tumour suppressor genes strengthened the case behind this supposition (Cohen, 1998). Neoplastic development bases itself on the existence of several genetic mutations, despite the number not being known. In most of the cases it is assumed to vary between tissues and between different species (Grisham et al., 1984; Cohen, 1998; Simons, 1995; Van Leeuwen and Zonneveld, 2001; Lutz, 2001; Gutiérrez and Salsamendi, 2001). During cell division, spontaneous genetic errors occur. It is estimated to happen at a frequency of around $10^5$ to $10^6$ through nucleotides and cell division. If the damage reaches a gene responsible for neoplastic development then the probability of developing cancer will be greater (Cohen, 1995).

A cancer is made up of billions of cells, all originating from an initial cell which multiplies clonally, escapes to apoptosis and accumulates genetic and/or epigenetic alterations which converge into a neoplastic cell (Trosko, 2001). The blocking of apoptosis in the face of significant genetic damage can ease the accumulation of aberrant cells and it can become a critical point in malignance pathogenesis (Nguyen-ba and Vasseur 1999, Qu et al., 2002).

Neoplasias can be classified as benign or malignant depending on their cellular characteristics. The constituent cells of a malign neoplasia show yet more changes in cell biology. They proliferate autonomously, differentiate themselves, invade adjacent tissues and frequently metastasize on tissues that are not related to the primary neoplasia (Hanahan and Weinberg, 2000; Shacter and Weitzman, 2002). Cells, which are part of benign neoplasias, grow more slowly, and in general, they do not disturb normal tissue function, unless they compress vital structures (Player et al., 2004).
3. Liver

Liver is the second largest organ of the body and first largest internal organ of the body. Liver is slightly pyramidal in shape and it lies under right ribs just beneath the right lung (Figure 1). It weighs about 1500 grams in normal adult human.

Unlike most of organs, liver has two sources of blood supply- the hepatic artery supplies oxygenated blood, portal vein bring nutrient rich blood from intestines. Liver is further divided into right and left lobes which are further sub divided into segments (Figure 2).

![The Liver](image1.png)

![Internal Anatomy of Liver](image2.png)

**Figure 1**

**Figure 2**
3.1. **Anatomy of Liver**

The liver is divided into 4 lobes: right, left, caudate, and quadrate. The right and left lobes are the largest, while the caudate and quadrate are smaller and located posteriorly. Two ligaments are visible anteriorly. Superiorly, the falciform ligament separates the right and left lobes. Inferior to the falciform ligament is the round ligament, which protrudes from the liver slightly. Also visible anteriorly on the most inferior portion of the right lobe is the gallbladder. Posteriorly, many more interesting structures are visible. The caudate lobe is located superiorly, approximately between the right and left lobes. Adjacent to the caudate lobe is the sulcus for the inferior vena cava. Just inferior to the caudate lobe is the porta hepatis, where the hepatic artery and hepatic portal vein enter the liver. The portal vein carries nutrient laden blood from the digestive system. Inferior to the porta hepatis is the bile duct which leads back to the gallbladder. Finally, the hepatic vein, where post-processed blood leaves the liver, is found inferior and adjacent to the sulcus for the inferior vena cava. The liver is held in place by a system of mesenteries posteriorly, and is also attached to the diaphragm via the falciform ligament. Additionally, most of the liver is covered by visceral peritoneum. (Guyton and Hall, 2006).

The basic functional unit of the liver is the liver lobule. A single lobule is about the size of a sesame seed and is roughly hexagonal in shape. The primary structures in a lobule include:

- Plates of hepatocytes form the bulk of the lobule
- Portal triads at each corner of hexagon
- Central vein
- Liver sinusoids that run from the central vein to the portal triads
- Hepatic macrophages (Kupffer cells)
- Bile canaliculi (little canals) - formed between walls of adjacent hepatocytes
- Space of Disse - a small space between the sinusoids and the hepatocytes

The portal triads consist of three vessels: a hepatic portal arteriole, a hepatic portal venule, and a bile duct. The blood from the arteriole and the venule both flow in the same direction, through the sinusoids toward the central vein, which eventually leads to the hepatic vein and the inferior vena cava. Secreted bile flows in the opposite direction, through the bile canaliculi away from the central vein, towards the portal triad, and exiting via the bile duct. As blood flows through the sinusoids and the space of Disse towards the central vein, nutrients are processed and stored by the hepatocytes, and worn out blood...
cells and bacteria are engulfed by the Kupffer cells. The lobule largely consists of hepatocytes (liver cells) which are arranged as interconnected plates, usually one or two hepatocytes thick. The space between the plates forms the sinusoid. A more functional unit of the liver forms the acinus. The functional acinus can be divided into three zones: 1) the periportal zone, which is the circular zone directly around the portal canal, 2) the central zone, the circular area around the central vein, and 3) a midzonal area, which is the zone between the periportal and pericentral zone. (Marieb, 2001)

3.2. Cells of the liver and functions

The liver has five cell types:

- Hepatocytes
- Kupffer cells
- Sinusoidal endothelial cells
- Bile duct epithelial cells
- Ito cells (Stellate cells)

3.2.1. Hepatocytes

Hepatocytes represent 60% of the liver’s cells, and about 80% of the liver’s total cell mass. Most of the liver’s synthetic and metabolic capabilities stem from the work of hepatocytes. Hepatocytes are arranged in plates only a single cell thick. Blood flowing toward the hepatic vein within the space of Disse passes both exposed surface areas of the hepatocyte plates, and toxins and nutrients within the blood are extracted by the hepatocytes (Heuman, 1997).

As mentioned above, hepatocytes accomplish almost all functions of the liver. These cells undergo numerous complex biochemical reactions per second to execute more than 500 vital functions of the liver (Monshouwer and Hoebe, 2003). Some of the important functions include (i) carbohydrate metabolism through storage of glycogen as well as performing of glycogenolysis and gluconeogenesis; (ii) production of many important proteins like serum albumin, fibrinogen, and the prothrombin group of clotting factors; (iii) role in lipid metabolism, by synthesizing and catabolising lipoproteins; (iv) production of bile, which is important for the digestion of dietary lipids and the excretion of hydrophobic substances; and (v) detoxification of xenobiotics like drugs or toxins as well as endogenous substances like ammonia through the urea cycle (Kmieć, 2001; Monshouwer and Hoebe, 2003).
3.2.2. **Liver sinusoidal cells – members of the mesenchymal compartment**

The liver is a highly perfused organ receiving approximately one quarter of total blood output from the heart which flows through four different vasculatures with distinct functions: the portal vein, the hepatic artery, the liver sinusoid and the hepatic vein. The high magnitude of metabolic activity in the liver is dependent on its dual blood supply via the hepatic artery (25%) and the portal vein (75%), which branch into hepatic arterioles respectively into portal venules that feed into the liver sinusoid. The liver sinusoid is a specific capillary network important for the exchange of various metabolic substances between hepatic blood flow and hepatic parenchymal cells (Enomoto et al., 2004; Le Couteur, 2008).

3.2.3. **Endothelial cells**

Endothelial cells (EC) account for the major cell population of the liver sinusoid (approximately 50% of all sinusoidal cells) and form a continuous lining of liver sinusoids separating the parenchymal cells from the sinusoidal blood. Liver sinusoidal endothelial cells are unique cells, which differ in several structural and functional aspects from other endothelial cells of the body. They are flat, elongated cells with a small cell body constituting the sinusoidal wall, which is characterized by open pores known as fenestrae and a small nucleus (Kmieć, 2001). Endothelial cells lack a basal membrane underneath the endothelium respectively along the space of Disse (subendothelial space) (Figure 3). The fenestrae function to filter fluids, solutes and particles that are exchanged between the sinusoidal blood and the hepatocytes, only substances smaller than the diameter of the fenestrae can pass the endothelium (Braet and Wisse, 2002). This fact plays an important role in hepatic lipoprotein metabolism as the endothelium acts as barrier for big triglyceride-rich chylomicrons while the smaller chylomicron remnants that can enter the space of Disse and reach the hepatocytes are metabolized (Fraser et al., 1995).
3.2.4. **Sinusoidal endothelial cells** are fenestrated (Latin for “windows”), meaning they have large pores that allow most proteins to pass freely through the sinusoidal endothelium into the space of Disse, where they can make direct contact with hepatocytes. The pores are also bi-directional, meaning that proteins created by the liver and other substances stored or processed by the liver can also be passed back into the blood. Sinusoidal endothelial cells function as a barrier against pathogenic agents and selectively filter for substances passing from the blood to parenchymal cells and vice versa. Along with Kupffer cells, they constitute the reticulo-endothelial system (RES) of the liver, where endothelial cells function as pinocytes and KCs as phagocytes of the RES (Smedsrød, 2004). The filtering effect of endothelial cells is enhanced by the clearance capacity of ECs based on receptor-mediated endocytosis. Especially, connective tissue macromolecules like hyaluronan, chondroitin sulphate or collagen α chain may be exclusively cleared from the blood circulation by ECs via receptor mediated endocytosis (Eriksson et al., 1983; Smedsrod et al., 1985a, Smedsrod et al., 1985b; Smedsrod, 2004). Furthermore, endothelial cells play an important role as part of the immune system. Besides Kupffer cells, dendritic cells and stellate cells, endothelial cells appear to be the fourth most predominant cell population in the liver that is important in antigen presentation (Knolle and Limmer, 2003). Additionally, endothelial cells also take part in the complex network of hepatocellular interactions through secretion of cytokines and growth factors (Kmieć et al., 2001).

3.2.5. **Kupffer cells**

Kupffer cells are macrophages that reside in the sinusoids. These cells help clear out old red blood cells and bacteria. They also break down heme (the iron-containing pigment in
hemoglobin) into bilirubin, which then becomes one of the chief pigments of bile. A later by-product of bilirubin gives faeces its characteristic brown colour (Heuman, 1997).

Kupffer cells (KC) are the resident macrophages of the liver and are located within the lumen of the hepatic sinusoids either attached to the luminal surface or inserted in the endothelial lining of sinusoids (Figure 4). Kupffer cells represent the largest population of macrophages in the body and are responsible for the clearing of sinusoidal blood from gastrointestinal derived bacteria and bacterial toxins. Besides their high phago- and endocytotic activity, Kupffer cells secrete various mediators which affect other mesenchymal cells and the parenchymal compartment of the liver in a paracrine manner (Kmieć, 2001). Kupffer cells are larger than ECs and have an irregular shape caused by numerous micrivilli, filopodia and lamellopodia which extend from the cellular surface and penetrate into the endothelial fenestrae to reach out into the space of Disse coming in contact with hepatocytes and stellate cells (McCuskey and McCuskey, 1990, Wisse, 1974). KCs are concentrated in the periportal region of the liver, which is a key location for monitoring blood entering the liver. However, KCs from the periportal zone are larger and have a higher endocytotic activity than mid-zonal and perivenous Kupffer cells. Whereas, perivenous Kupffer cells are more active in cytokine production and have a higher cytotoxic activity (Naito et al., 2004; Kolios et al., 2006).

Figure 4: Scanning microscopy of a Kupffer cell in the sinusoid (Naito et al., 2004).

Kupffer cells are constantly exposed to gut-derived mediators known to activate macrophages. Lipopolysaccharides (LPS) - glycolipids found on the membrane of all gram-negative bacteria - are the most important bacterial endotoxins, which stimulate KCs via binding to the toll-like receptor 4 (TLR-4) followed by the release of inflammatory
cytokines like TNF-α and IL-1 as well as reactive oxygen intermediates (Su, 2002). The clearance of various particles from the blood flow also causes release of biologically active substances including proteases, cytokines and reactive oxygen species (Vrba and Modriansky, 2002). Thus, oxidative burst i.e. the uptake of oxygen and its transformation in ROS that on one hand is essential for host defence, but on the other hand may be deleterious for the healthy tissue in the vicinity.

Besides typical macrophage functions KCs, are responsible for the uptake of senescent erythrocytes from the blood and subsequent degradation of heme to bilirubin (Hirano et al, 2001). KCs also act as antigen presenting cells due to the expression of MHC I- and MHC II molecules as well as the expression of the co-stimulator molecules CD40 and CD80. Furthermore, they are involved in intrahepatic immunosuppression and induction of immunological tolerance (Kmieć, 2001; Selmi et al., 2007). Generally, KCs are implicated in the pathogenesis of numerous liver diseases including alcoholic and non-alcoholic liver disease, liver fibrosis and hepatocellular carcinoma; as in liver injury, KCs when activated, release biologically active substances including inflammatory cytokines, superoxide as well as eicosanoids and proteolytic enzymes, which promote the pathogenic process (Kolios et al., 2006).

3.2.6. Stellate Cells

Stellate cells (SC), also known as Ito cells, vitamin A-storing cells, perisinusoidal cells, lipocytes or liver-specific pericytes, have spindle-shaped cell bodies with elongated nuclei and characteristic cytoplasmic processes which provide contact to HC, EC and other SC (Figure 5) (Friedman, 2008; Geerts, 2001). Another typical morphological property of SCs is the presence of fat droplets in their cytoplasm containing retinoids, triglycerides, cholesterol and free fatty acids (Wake, 1980).

Hepatic stellate cells are heterogeneous cells and are characterized by high plasticity depending on whether the liver is healthy or diseased. In normal livers, they are “quiescent” with lipid droplets in the cytoplasm, a low proliferation rate and low synthetic activity; whereas in chronically diseased livers especially in case of fibrosis, the SCs are “activated” (Kmieć, 2001; Friedman, 2008). This myofibroblast-like phenotype is characterized by loss of lipid droplets, increased cell proliferation and enhanced synthesis of extracellular matrix components (Kmieć, 2001; Friedman 1993). Progressive activation of stellate cells can be studied in primary cultures of SCs. When SCs are plated on standard tissue culture plastic, they acquire an activated phenotype (Friedman, 1992) and formation of “stress fibers” is observed (Yee, 1998). This phenotypic transition of SCs is also
accompanied by any change in the expression of various proteins like receptors and cytokines as well as proteins of the extracellular matrix (Kristensen et al., 2000).

![Stellate cell (blue) situated in the space of Disse](image)

**Figure 5:** Stellate cell (blue) situated in the space of Disse (Friedman, 2008)

SCs are also important mediators of intercellular communication through secretion of various cytokines and growth factors like HGF, IGF, vascular endothelial growth factor (vEGF), interleukin 6 and 10 (Kmieć, 2001; Friedman, 2008). Additionally, activated SCs secrete TGF-β which is an important promoter of fibrogenesis (Gressner and Weisskirchen, 2006).

3.2.7. **Bile duct epithelial cells** line the interlobular bile ducts within the portal triads.
4. Epidemiology of hepatocellular carcinoma (Hepatocellular carcinoma)

Hepatocellular carcinoma (HCC) belongs to the group of epithelial cancers and represents with a frequency of about 85%, the most common primary liver cancer (McKillopp et al., 2006). HCC has become the fifth most common malignancy and the third leading cause of cancer death worldwide exceeded only by cancers of the lung and the stomach (Caldwell and Park, 2009; Gomaa et al., 2008). The estimated incidence may be located between 500,000 -1,000,000 new cases per year (Bosch et al, 2004; Gomaa et al., 2008; Sherman 2005), and is characterized by a wide geographic variation; it ranges from less than 10 cases / 100,000 in the USA and Western Europe to 50 - 150 cases / 100,000 in parts of Africa and Asia (Blum and Spangenberg, 2007). This enormous discrepancy can be explained by the most important risk factors of hepatocellular carcinoma, which are predominant in developing countries like hepatitis B virus (HBV) infection or exposure to aflatoxin B1 contaminated food (Llovet et al., 2003). On the other hand the incidence of HCC in Western industrialized countries and also in Austria is increasing during the last 20 years (El-Serag and Mason, 1999; Khan et al., 2002). One cause for this development may be the increase in hepatitis C virus (HCV) infections, but also non-alcoholic fatty liver disease due to obesity has been recognized as independent risk factor (Caldwell and Park, 2009). Incidence of HCC is not only characterized by regional differences, but also by sex-dependent ones, as the incidence in men is about twice as high as in women. Explanations for this phenomenon may be hormonally-based differences in the inflammatory response of the liver towards viral infections or other cell-damaging factors (Caldwell and Park, 2009; Naugler et al., 2007).

The most prominent etiological factors associated with hepatocellular carcinoma are chronic hepatitis B and C viral infections, chronic ethanol abuse and intake of aflatoxin B1-contaminated food (McKillop et al., 2006). Recently also obesity has been recognized as a predisposing factor for the development of liver cancer (Qian and Fan, 2005). Apart from aflatoxin B1, where no clear connection between exposure and development of cirrhosis can be assessed, all etiological factors may cause the development of chronic hepatitis followed by cirrhosis. Cirrhosis and chronic hepatitis infections represent the underlying aetiology in more than 80 % of HCC cases (McKillop et al., 2006). Whereas hepatitis viruses B and C exhibit a direct oncogenic potential (Di Bisceglie, 2009; Levrero, 2006), alcohol mediated development of HCC is based on several mechanisms such as the genotoxic effect of acetaldehyde (a metabolite of ethanol) as well as DNA damage induced...
by reactive oxygen species (ROS) and lipid peroxidation products (Seitz and Stickel, 2007). Despite of various etiological factors in hepatocarcinogenesis, the development of HCC is invariably associated with chronic liver injury and the subsequent inflammatory reaction triggering a sustained wound healing process.

5. **Types of liver cancer**

5.1. **Hepatocellular carcinoma (HCC)**
Hepatocellular carcinoma begins in the hepatocytes. This is the most common form of liver cancer in adults. It has different growth patterns. Some start as a single tumor that grows larger. Only late in late stages it spread to other parts of the liver. Others seem to start in many spots throughout the liver, not as a single tumor. This is most often seen in people with ongoing liver damage (cirrhosis) or any liver injury.

5.2. **Hepatoblastoma**
Hepatoblastoma is the most common in children younger than 20 years old (London and McGlynn, 2006) and represents 1% of malignancies with a peak incidence of 11.2/1,000,000 during infancy (Ahrens et al., 2007). Etiology of hepatoblastoma is still unknown. Current knowledge on the cause includes Beckwith–Wiedemann syndrome, hemohypertrophy, familial adenomatous polyposis, and Gardner’s syndrome. About 70% of children with this disease have good outcomes with surgery and chemotherapy. The survival rate is greater than 90% for early-stage disease.

5.3. **Cholangiocarcinoma or Bile duct cancer**
Bile duct cancers account for 1 or 2 out of every 10 cases of liver cancer and accounts for about 3% of gastrointestinal cancers worldwide. These cancers start in the small tubes (called bile ducts) that carry bile to the gallbladder. (Khan et al., 2005). In most regions of the world the incidence are in the range 0.2–2/100,000, the incidence is much higher in areas where liver fluke infestation is common, such as North-East Thailand (London and McGlynn, 2006).

5.4. **Hepatic Angiosarcoma**
Hepatic angiosarcoma is a rare mesenchymal tumor of the liver that starts in the blood vessels of the liver, which usually presents in elderly men (Rademaker et al., 2000). Workers who are occupationally exposed to vinyl chloride are at an increased risk to angiosarcomas. These tumors grow quickly. Often by the time they are found they are too widespread to be removed. Treatment may help slow the disease, but these cancers are usually very hard to treat.

5.5. **Metastatic cancer or secondary liver cancer**
This type of cancer is found in liver but it did not start in there, but started somewhere else (like the colon, breast, or lung) and spread to the liver. Even though these cancer cells are in the liver, they still look and act like cancer cells from the part of the body that they came from.

6. **Stages of Hepatocarcinogenesis**

Hepatocarcinogenesis is a multi-step process that contains the succession from preneoplastic lesions to malignant neoplasms associated with numerous genetic and epigenetic alterations (Wong and Ng, 2007). Initiation, promotion and progression are the main three stages involving in the carcinogenesis process.

6.1. **Initiation**

Initiation involves irreversible alteration in the cellular DNA by a carcinogen resulting in the activation of oncogenes and the inactivation of tumor suppressor genes. A normal cell can be initiated by a single gene mutation caused in most cases by environmental genotoxic agents such as chemicals, radiation, and viruses. Oncogenes can also be activated by chromosomal translocations and gene amplifications (Wattenberg, 1996). Initiated cells are difficult to distinguish morphologically and phenotypically from their normal counterparts and the molecular mechanisms too are difficult to identify. Depending on the location of the mutation in the genome, an initiated cell could need further modifications or requires additional cellular modifications to produce malignant hepatic tumours (Pitot and Sirica, 1980). Initiation can occur after a single genotoxic exposure, or spontaneously through DNA repair infidelity. If the genetic mutation is fixed, then the initiated cell can remain dormant for a period of time. These quiescent initiated cells are very highly susceptible to be transformed into malignant cells (Klaunig and Kamendulis, 2004). Initiating agents form adducts with the DNA. This adduct formation leads to the alterations in the various signal transduction pathways involved in the control of cell growth, differentiation and other cellular functions. Several classes of genes appear to be appropriate target DNA damaging carcinogens. Alkylation agents causes the addition of alkyl group to DNA. The directly acting alkylating agents induce preferential binding to highly nucleophilic centres such as the N7 position of guanine. The position of adduct formation in the DNA and its chemical and physical properties will determine the types of mutations induced (Essigmann and Wood, 1993).

6.1.1. **Diethylnitrosamine (DEN) as an initiating agent**
Diethylnitrosamine (DEN) also known as N-nitrosodiethylamine is a potent initiator of hepatocarcinogenesis and is widely used as an initiator of carcinogenesis in experimental models (Bhosale et al., 2002). DEN has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals (Ramakrishnan et al., 2006; Valko et al., 2006). Exposure to DEN occurs due to the use of smoked food, tobacco products, cosmetics, pharmaceutical products and agricultural chemicals (Verna et al., 1996; Hecht, 1997).

DEN administration to mice, intraperitoneal injection or orally to mice leads to the development of various tumours including liver, the gastrointestinal tract, skin, the respiratory tract and hematopoietic cells. Many investigators have used DEN to induce liver tumors in mice by injecting DEN i.p. into weaning mice at 2 weeks after birth, giving rise to hepatic tumors nearly 8 months later (Naugler et al., 2007; Fan et al., 2010). Since DEN does not itself exert carcinogenicity, it needs to be bio-activated by cytochrome P450 (CYP) enzymes in the liver, resulting in DNA-adducts, through an alkylation mechanism (Verna, 1996). It is metabolized in two steps to first yield a hydroxylated form of DEN which later splits to produce an electrophilic ethyl ion. The CYP 2A6 and 2E1 isoform of cytochrome P450 were responsible for the metabolism of DEN to its ultimate carcinogenic form, the ethyl ion (Anis et al., 2001; Chakraborty et al., 2007). The ethylation of the DNA by DEN causes a mutation of the codon 61 in the Ha-ras gene in the mouse liver (Bauer-Hofmann et al., 1992). These alkylation adducts can be removed by a DNA repair gene O^6^-methylguanine-DNA methyltransferase (MGMT), also known as O^6^-alkylguanine-DNA alkyltransferase (Jacinto and Esteller, 2007). Recently, Kang et al demonstrated that CYP2E1 deficient mice show lower tumor incidence and multiplicity compared with wild-type mice for DEN-induced hepatocarcinogenesis (Kang et al., 2007), confirming the essential role of CYP 2E1 in the activation of DEN, although several other CYP enzymes are proposed to catalyze DEN bio-activation in-vivo (Verna, 1996). Experimental, clinical and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer.

6.1.2. Properties of initiated cell

Minimal operational properties of tumour-initiating cells (Clarke et al., 2006) are:

- Tumour cells that have the ability to re-grow the tumour from which they were isolated or identified.
- Tumour-initiating cells are viewed at the apex of the tumour hierarchy, which highlights the role of aberrant differentiation in tumorigenesis.
Multipotency of lineage differentiation is likely to be a frequent, but not a necessary, property of tumour-initiating cells.

6.2. Promotion
Promotion follows initiation and involves the process of gene activation, such that the latent phenotype of the initiated cell becomes expressed through cellular selection and clonal expansion of initiated cells into islands of altered hepatocytes defined as focal lesions (Diaz-Cano, 2012). The cells with altered focal lesions have altered morphologic, enzymatic and proliferative parameters as compared with normal hepatocytes. Several studies have demonstrated that number of hepatic lesions and volumes of lesions were decreased after the removal of the promoting stimulus (Slaga, 1983b). Promotion can occur through a variety of mechanisms, including toxicity, terminal differentiation or mito-inhibition of the non-initiated cells, and mitogenesis of the initiated cells (Slaga, 1984).

6.2.1. Tumor promoting factors in Hepatocarcinogenesis
The span of the available data, as well as the multistage nature of tumor promotion, suggests that this process, which is now thought to occur in most tissues in which cancer can be induced or in which it occurs spontaneously, may involve the interaction of a number of endogenous factors as well as environmental factors such as chemicals, radiation, viruses, bacteria, and diet and nutrition, thus unifying all current areas of cancer research (Moore and Kitagawa 1986; Slaga et al., 1995). After a sufficient initiating treatment, the carcinogenic process can evolve naturally or operationally be modulated. In liver carcinogenesis, the promoting factors include chemical or viral carcinogens (2-acetylaminofluorene, diethylnitrosamine, pentobarbital etc.), non-genotoxic xenobiotics (drugs, pesticides, food additives, and contaminants), endogeneous compounds (harmones, growth factors) dietary factors (mycotoxins, polycyclic aromatic hydrocarbons or aromatic amines present in food) and surgery (partial hepatectomy).

6.2.2. Acetylaminofluorene (2-AAF) as tumour promoter
2-Acetylaminofluorene (2-AAF), an aromatic amide, generally not present in the environment was originally developed as an insecticide, but presently among one of the most intensively studied liver carcinogen (Neumann et al., 1990; Wilson et al., 1941). 2-acetylaminofluorene (2-AAF) requires metabolic activation which leads to the formation of its ultimate carcinogenic metabolite. This metabolic activation of 2-AAF involves two necessary steps to form the reactive metabolites (Schrenk et al., 1994) (Figure 6). The initial reaction, N-hydroxylation, is a cytochrome dependent phase I reaction, whereas the second
reaction, resulting in the formation of the unstable sulfate ester, is a phase II conjugation reaction that results in the formation of the reactive intermediate. Another phase II reaction, glucuronide conjugation, is a detoxification step, resulting in a readily excreted conjugation product (Miller and Miller, 1981).

In some animal species, 2-AAF is known to be carcinogenic, whereas in other species it is non-carcinogenic. The species and sex specific carcinogenic potential of 2-AAF is correlated with the ability of the organism to sequentially produce the N-hydroxylated metabolite followed by the sulfate ester. Therefore in an animal such as the guinea pig, which does not produce the N-hydroxylated metabolite, 2-AAF is not carcinogenic. In contrast, both male and female rats produce the N-hydroxylated metabolite, but only male rats have high rates of tumour formation. This is because male rats have up to 10-fold greater expression of sulfotransferase 1C1 than female rats, which has been implicated in the sulfate conjugation of 2-AAF resulting in higher production of the carcinogenic metabolite (Hodgson, 2004).

![Bioactivation of 2-acetylaminofluorene](image)

**Figure 6:** Bioactivation of 2-acetylaminofluorene (Hodgson, 2004)

Microsomal generation of reactive oxygen by redox cycling from 2-AAF was proposed to be the possible tumor promotion mechanisms of 2-AAF (Hillesheim et al., 1995). It is also known to interact with energy metabolism (Ambs and Neumann, 1996), an increase in glutathione in neoplastic nodules has also been described (Rommi et al., 1985). 2-AAF exerts promotional effects by stimulating the growth of focal cells rather than by suppressing proliferation of normal hepatocytes (Tiwawech et al., 1991). 2-AAF administration in diet leads to the aberrant expression of GST-P, c-FOS and increased apoptosis at early stages of rat hepatocarcinogenesis (Hadjiolov et al., 1995). This
observation supports the proposal of oxidative stress and energy impairment in the mitochondrial of periporal hepatocytes in the rat liver (Klöhn et al., 1995). 2-AAF induces p21^waf1/cip1 expression in non-neoplastic hepatocytes, but this expression is not observed in neoplastic hepatocytes, which leads to the phenomenon of selective growth of preneoplastic lesions (Watanabe et al., 1998). Thus 2-AAF acts as a selective pressure inducing agent for the enhancement of proliferation in altered hepatic neoplasm. Metabolites of 2-AAF are mitotic inhibitors and inhibits the proliferation of normal hepatocytes, but the altered cells are not able to metabolize 2-AAF and hence are selectively protected from the toxic effects of the 2-AAF metabolites and continue to proliferate (Roomi et al., 1985).

6.2.3. Ornithine decarboxylase (Marker of tumor promotion)
Ornithine decarboxylase (ODC) is the first and rate limiting step in the biosynthesis of polyamines, which are ubiquitous intracellular bases required for normal and neoplastic growth (Heby, 1981). This enzyme has been implicated as an essential biomarker of cellular proliferation and tumour development (O'Brien, 1976; Boutwell, 1983). Increasing evidence indicates that ODC and polyamines have an important role in the regulation of cell proliferation and in the development of cancer (Flamigni et al., 1999). Growth induction of normal cells is known to be accompanied by a rapid transient increase in ODC activity (Iwata et al., 1999; Gilmour and O'Brien, 1989). Cell transformation induced by oncogenes such as v-src, neu, and ras has been shown to be associated with constitutively elevated ODC activity (Flamigni et al., 1999; Sistonen et al., 1989; Auvinen et al., 1992; Holtta et al., 1993). The up-regulation of ODC is considered essential for cell transformation. Studies have demonstrated the role of ODC in the development of chemically induced liver cancer (Machishi et al., 1995)

6.3. Progression
The last step leading to cancer is called progression. Progression involves genetic damage that results in the conversion of benign tumors into malignant neoplasms capable of invading adjacent tissues and metastasizing to distant sites. The additional genetic alterations thought to be required for neoplastic progression often occur faster than would be expected from the statistics of accidental genotoxic insults due to so called genetic instability. The concept of genetic instability implies that while environmental genotoxic agents generally cause cancer initiation, the additional mutations required for neoplastic progression may be attributed to endogenous reactions and factors such as detoxification
and removal of damaged cells by programmed cell death (Nicolson, 1987). Genetic instability may happen due to the errors in DNA replication, spontaneous hydrolytic alterations of DNA such as depurination and deamination in combination with an impaired ability of premalignant cells to repair DNA damage or due to oxidative DNA damage (Pitot, 1989). Modified DNA bases, especially 8-hydroxy-2-deoxyguanosine, produced by oxygen-free radicals have been implicated in the genesis of cancer (Thompson, 2004). The importance of free radicals in radiation carcinogenesis and oxygen-free radicals and electrophiles in chemical carcinogenesis is also well recognized (Pitot, et al., 1978).

Figure 7: The multiple steps of hepatocarcinogenesis (Thorgeirsson and Grisham, 2002)

6.3.1. Tumour proliferation markers (Ki67 and PCNA)

Ki67-Markers of cellular proliferation have been used for prognostication in several and hold some promise as prognosticators in hepatocarcinogenesis. A proliferative index can be determined by immunohistochemistry using the monoclonal antibody Ki67, which results with the large nuclear Ki67 protein required for cell proliferation (Brown and Gatter, 2002). Ki67 is expressed in the G1, G2, S, and M phases of the cell cycle but is not expressed in resting cells (G0). Ki67 expression has been identified as a prognostic marker in various types of cancers (Baak et al., 2009; Brown and Gatter, 2002).

Ki67 has been reported as an independent predictor of rapid tumor recurrence in patients who underwent orthotopic liver transplant (Guzman et al., 2005). In benign liver from chronic HBV and chronic HCV patients, Ki67 positivity correlated independently with transaminase levels (ALT) and etiology of liver disease, suggesting that these factors may be confounding tumor staining patterns (Farinati et al., 1996).
**PCNA** - a commonly used marker of cellular proliferation. Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in DNA synthesis and repair. PCNA is a nuclear protein that is synthesized in G1-S-phase of the cell cycle. It is an accessory factor for DNA polymerases (Δ and Ε) and functions as a DNA sliding clamp. PCNA expression is reported to predict tumor recurrence, especially for small hepatocellular carcinoma (Ng et al., 1994; Suehiro et al., 1995), and is associated with venous invasion (Kitamoto et al., 1993).

PCNA is required for eukaryotic DNA synthesis, replication, and repair and is expressed at high levels in cycling cells (Kawakita et al., 1992). Other PCNA cellular functions include Okazaki fragment joining, DNA methylation, and chromatin assembly (Kelman, 1997; Jonsson and Hubscher, 1997). Expression of PCNA dramatically progressed from chronic hepatitis infection to hepatocellular carcinoma (Kawakita et al., 1992). PCNA can be also assayed by immunohistochemistry and is usually scored as a percentage – delta (Δ%), and its expression is related to DNA synthesis and replication.

### 6.4. Tumor metastasis

As the tumor progression advances, the cells lose their adherence property, detach from the tumor mass and invade the neighbouring tissues. The detached cells also enter the circulating blood and lymph and are transported to other organs/tissues away from the site of the primary growth and develop into secondary tumors at the new sites. These form the distant metastases, resulting in widely spread cancers. Cancer metastasis consists of a number of steps; the main steps are common for all tumors. The progress of the neoplastic disease depends on metastatic changes that facilitate: (a) invasion of local normal tissues, (b) entry and transit of neoplastic cells in the blood and lymphatic systems, and (c) the subsequent establishment of secondary tumor growth at distant sites (Hart and Saini, 1992, Takeichi, 1993).

Many of the steps in tumor metastasis involve cell-cell and cell-matrix interactions, involving specific cell surface molecules. Malignant cells are thought to have reduced ability to adhere to each other, so that they detach from the primary tumor and invade the surrounding tissues. The behaviour of tumor is influenced by the cell adhesion molecules, one of the most important of which is cadherins (Takeici 1991). Animal studies have shown that a down regulation of E-cadherin expression, resulting in lower levels, correlated with metastatic behaviour in-vivo, suggesting that cadherins function as invasion suppressor gene products (Vleminckx et al., 1991).
It is the metastatic process and tumors spreading that are mainly responsible for the lethal effects of many common human tumors. In many cases gene mutations are believed to be the driving force for tumor metastasis, with the development of tumor vasculature playing an important role in the disease progression (Folkman, 1995).

7. Different models of Liver cancer

There are currently available at least six types of models for the study of hepatocarcinogenesis including the study of nodules. These are as follows:

7.1. Long Term Continuous Exposure to a Carcinogen

This type of model was used with many different carcinogens since 1933. It is still used in many studies. Its major disadvantages are:

(a) no synchrony of lesion development is seen and therefore it is very difficult to study the genesis, options and fates of any single type of lesion including the various types of nodules; and

(b) The nodules generated are often small and of many different sizes.

7.2. Intermittent Chronic Exposure

This model was first developed by Reuber in 1965 and subsequently modified by Epstein et al., 1967, and later by Teebor and Becker. The carcinogen containing diet is fed intermittently with intervening periods of control diet. Large nodules are generated and a distinction between “regression” or reversibility and persistence of nodules was highlighted.

7.3. Resistant Hepatocyte Model (RH Model) or Solt Farber model of HCC

This model was developed by Solt, Farber and their colleagues in 1977. It utilizes a single dose of carcinogen, coupled with cell proliferation to initiate and a two week exposure to dietary 2-acetylaminofluorene (2-AAF), plus partial hepatectomy (PH) to rapidly select for resistant hepatocytes that proliferate to form nodules. Nodules appear synchronously as a cohort.

This model of hepatocarcinogenesis consists of three main components: an initiator (Diethylnitrosamine), a selective growth inhibitor (2-acetylaminofluorine), and a generalized potent growth stimulus (Partial Hepatectomy). DEN is known to induce liver cancer in the rats slowly with a single oral, intravenous, or intraperitoneal administration. DEN was given to rats at a dose of 200mg/kg body weight. Following a two week period of recovery from the initial cell damage, the animals were fed a standard basal diet containing 0.02% 2-AAF for six weeks and were subjected to 67% partial hepatectomy.
(PH) at third week. Feeding of 2-AAF diet for about one week before PH is sufficient to induce basophilic foci. The strong selection drive (2-AAF + PH) used in this model leads to an intensive proliferation of the nodule cells resulting in very early appearance of the nodules. It also favours the developmental phase since more tumours are known to arise in this system than after administration of DEN alone. At the end of eight weeks of initiation, half the numbers of animals from each group were sacrificed for histopathological studies and the remaining of the animals were left for the progression till 24 weeks. Solt and Farber model of HCC is very efficient one to study the mechanism of carcinogenesis and factors affecting hepatocarcinogenesis. Moreover, this model is highly useful in investigating the chemopreventive potential of various natural or synthetic agents, factors affecting liver cancer development in humans and synergistic effects of xenobiotics in rats. Ito and his colleagues have modified this model with the use of many other carcinogens for selection other than 2-AAF (Ito et al., 1980).

7.4. Chronic Enzyme Induction Model (CEI Model)
This was first developed by Peraino and colleagues in 1971 and has been utilized by Pitot and others. In this model, Initiation is brought about by a single or brief exposure to a carcinogen and then is promoted by a long term exposure (about 6 months) to dietary phenobarbital, DDT, a-hexachlorocyclohexane, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other liver enzyme inducers. The major disadvantage of some of these models is the slow development of focal proliferations and the general lack of synchrony in the appearance and growth of nodules and other focal lesions.

7.5. Choline-Methionine Deficient Model
This has been developed by Lombardi, Shinozuka and their coworkers with earlier work by Newberne and coworkers. Initiation is by single exposure to carcinogens and promotion is by feeding a choline devoid diet for many months. The system appears to require further refinement for synchrony of nodule development to be achieved.

7.6. Orotic Acid Model
This model is being developed by Columbano, Sarma, Rajalakshmi, Rao and their colleagues (Columbano et al., 1982). Initiation is by a single dose of a carcinogen and promotion by feeding a diet containing orotic acid. It appears that the nodules develop quite synchronously, even though the rate is slower than in the resistant hepatocyte model.

8. Hepatocarcinogenesis and inflammation
Clinical observations support the idea, that there is a strong association of chronic inflammation in carcinogenesis. (Balkwill et al., 2005; Coussens and Werb, 2002). Reduction in the cancer risk associated with the long term use of non-steroidal anti-inflammatory drugs (NSAIDs) strengthens the proposed link between inflammation and cancer (Thun et al., 2002). Association of carcinogenesis and inflammation is also supported by the increased risk of cancer development by several non-infectious causes of chronic inflammation such as, cigarette smoke, asbestos and silica (Manning et al., 2002).

In addition to epidemiological data that link inflammation with cancer, polymorphisms in the inflammatory genes including tumour-necrosis factor-α (TNF-α), interleukin-1 (IL-1) and Toll-like receptors (TLRs) are reported to be associated with non-Hodgkin’s lymphoma, liver cancer, stomach cancer and prostate cancer, respectively (Karin and Greten, 2005).

### 8.1. Role of Cyclooxygenase-2 (COX-2) in hepatocellular carcinoma

Cyclooxygenase (COX), known as PG synthase, catalyzes the metabolism of arachidonic acid to PGs and thromboxanes (Smith et al., 1996), and two isoforms, COX-1 and COX-2, have been identified. COX-1 is constitutively present in many cell types and is responsible for various cytoprotective prostanoids in a number of organs, such as the gastric mucosa and the kidneys, whereas COX-2 is usually absent under basal conditions but inducible in certain cells by mitogens, cytokines, and other factors (Hla and Neilson, 1992; Jones et al., 1993).

The evidence that COX-2 may be a logical therapeutic target in HCC comes from studies that showed over expression of COX-2 in patients with HCC (Kondo et al., 1999; Koga et al., 1999; Bae et al., 2001; Leng et al., 2003; Cervello et al., 2005). COX-2 expression is generally higher in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver, suggesting that COX-2 may be involved in the early stages of hepatocarcinogenesis (Koga et al., 1999; Bae et al., 2001; Cervello et al., 2005). In addition, a significant correlation between COX-2 expression and active inflammation in the adjacent noncancerous liver has been reported (Morinaga, 2002), and increased expression of COX-2 in noncancerous liver tissue was significantly associated with shorter disease-free survival in patients with HCC (Kondo et al., 1999). This result is of great importance from a clinical point of view, as it suggests that COX-2 expression may play an important role in the relapse of HCC after surgery.

Furthermore, we recently reported that COX-2 expression in the tumor tissue was significantly correlated to the presence of inflammatory cells, macrophages and mast cells
(Cervello et al., 2005). However, COX-2 expressing cells and the number of both types of inflammatory cells decreased with progression of the disease, suggesting their possible involvement in the early stages of hepatocarcinogenesis.

The decrease in COX-2 expression during tumor progression as observed in HCC is unusual. A possible explanation for this different behavior pattern is that, in some cell types, COX-2 overexpression may cause a growth disadvantage, as suggested by Trifan (Trifan et al., 1999), who reported that COX-2 overexpression may induce cell cycle arrest in a variety of cell types.

8.2. **NF-κB and its role in liver cancer**

Several pro-inflammatory cytokines and chemokines, such as TNF, IL-1, IL-6 and IL8, all of which are encoded by target genes of the IKK-β (inhibitor of NF-κB (IκB) kinase-β)-dependent NF-κB-activation pathway, are linked with tumour development and progression in humans and mice (Balkwill and Mantovani, 2001). Furthermore, many oncogenes and carcinogens can lead to the activation of NF-κB, whereas various chemicals with known chemopreventive properties can interfere with NF-κB activation (Bharti and Aggarwal, 2002). Interestingly, recent animal studies provide strong evidence that, IKK-β-dependent NF-κB-activation pathway has direct molecular link between inflammation and carcinogenesis (Karin et al., 2002), and indeed a crucial mediator of tumour promotion (Greten et al., 2004 Pikarsky et al., 2004)

There are two distinct NF-κB-activation pathways: the classical pathway and the alternative pathway (Bonizzi and Karin, 2004) (Figure). Even though both pathways can affect tumour development, but most of the current research relates to the pro-carcinogenic functions of the classical pathway. This pathway is activated by bacterial and viral infections, as well as pro-inflammatory cytokines, all of which activate the IKK complex. This complex is composed of two catalytic subunits, IKK-α (also known as IKK1) and IKK-β (also known as IKK2), and a regulatory subunit, IKK-γ (also known as NEMO). The IKK complex phosphorylates
Figure 8: Signalling pathways that lead to the activation of different nuclear factor-κB transcription factors and the biological consequences of these pathways. (Karin and Greten, 2005)

NF-κB-bound IkBs, thereby targeting them for proteasomal degradation and liberating NF-κB dimers that are composed of REL-A (also known as p65), REL (also known as cREL) and p50 subunits to enter the nucleus and mediate transcription of target genes (Hayden and Ghosh, 2005). This process mostly depends on the catalytic subunit IKK-β, which carries out IkB phosphorylation (Ghosh and Karin, 2002). Interestingly, in this pathway, especially in macrophages, the IKK-α subunit has a negative-regulatory role by
phosphorylating the NF-κB subunits REL-A and REL on sites that accelerate their nuclear turnover, thereby contributing to cessation of the NF-κB-mediated gene-induction response (Lawrence et al., 2005).

The alternative NF-κB-activation pathway involves the upstream kinase NF-κB-inducing kinase (NIK) - which activates IKK-α homodimers, independently of either IKK-β or IKK-γ - leading to the phosphorylation and processing of p100, in response to certain members of the TNF family (Bonizzi and Karin, 2004; Hayden and Ghosh, 2005). The two pathways mediate different immune functions because they switch on different gene sets (Bonizzi and Karin, 2004).

The association of the classical NF-κB-activation pathway to acute inflammation and cell survival mechanisms is well documented. Due to the variety of target genes of the classical pathway, which include genes encoding mediators of inflammation, cytokines, chemokines, proteases and inhibitors of apoptosis, it has been proposed that classical NF-κB activation might link inflammation to tumour promotion and progression (Karin et al., 2002). Several studies, on chemically induced liver cancer, throws further light on this associative link between cancer and inflammation and demonstrated that, even in chemically induced carcinogenesis, in the absence of chronic inflammation, inflammatory processes in non-malignant cells are important determinants of tumour development (Pikarsky et al., 2004; Maeda et al., 2005).

8.3. Inflammation associated liver cancer and involvement of NF-κB

Two distinct mechanisms have been ascribed to the tumour promoting effects of IKK-β and NF-κB. The first mechanism involves increased survival of initiated and dysplastic cells in MDR2 (Multidrug Resistance 2 Gene)- knockout mice, a genetic model of cholangitis (bile-duct inflammation) that is caused by bile acid and phospholipid accumulation, which leads to the appearance of HCC within 8-10 months of birth (Mauad et al., 1994). In this model, inactivation of NF-κB between 7 and 14 months after birth, through expression of a non-degradable IκBα variant (known as the IκB super-repressor) under the control of a promoter that is highly active in hepatocytes, blocked tumour development. This effect was seen only when NF-κB was inhibited for a considerable duration in late tumour promotion and progression, but when NF-κB was inhibited for the first 7 months of life, during the early phases of initiation and tumour promotion, no beneficial effect was observed (Pikarsky et al., 2004). In Mdr2−/− mice, TNF expression is up regulated in the non-hepatocyte fraction of the liver (that is, in inflammatory and
endothelial cells), and this causes NF-κB activation in hepatocytes with cell-surface TNF receptors, through the classical pathway. The mechanism that underlies induction of TNF expression in this model, however, is not known. When mice were treated, after 7 months of age, with neutralizing antibodies specific for TNF, apoptosis of hepatocytes was induced as effectively as by inhibiting NF-κB between 7 and 14 months. In both cases, NF-κB target genes that encode known anti-apoptotic regulators, such as GADD45β (growth arrest and DNA-damage-inducible 45β) and BFL1 (also known as A1; a B-cell lymphoma 2 (BCL-2)-related protein), were inhibited, implying that TNF-dependent NF-κB activation drives tumour promotion in this model by inhibiting hepatocyte apoptosis.

8.4. Role of Nuclear factor kappa B (NF-κB) in chemically (DEN) induced hepatocarcinogenesis

Diethylnitrosamine (DEN), a known chemical carcinogen leads to the initiation of hepatocytes or to the death of hepatocytes through necrosis or apoptosis or both. In the case of a deficiency in IKK-β [inhibitor of nuclear factor-κB (NF-κB) kinase-β], necrotic cell death of DEN exposed hepatocytes is augmented by increased accumulation of reactive oxygen species and sustained activation of JUN amino-terminal kinase (JNK). This leads to the release of certain unknown cellular constituents, possibly HMGB1 (High Mobility Group Box 1 Protein), S100 calcium binding proteins, heat-shock proteins or purine metabolites, that activate IKK-β and NF-κB in adjacent Kupffer cells. Activated Kupffer cells then release pro-inflammatory cytokines, such as tumour-necrosis factor (TNF) and interleukin-6 (IL-6), and stimulate the production of hepatocyte growth factor by stellate cells, which together stimulate the proliferation of surviving, mutated hepatocytes (Maeda et al., 2005; Zeh and Lotze, 2005)
Figure 9: The role of nuclear factor-κB in chemically induced hepatocellular carcinoma: coupling necrotic cell death to compensatory proliferation through Kupffer-cell activation (Karin and Greten, 2005).

8.5. Role of Interleukin-6 in hepatocarcinogenesis

Interleukin-6 (IL-6) constitutes a pro-inflammatory cytokine, which may be an important link between chronic liver diseases and hepatocellular carcinoma. It has been observed that patients suffering from diverse chronic liver diseases including alcoholic liver disease, hepatitis B and C infections (Naugler and Karin, 2008) as well as from steatohepatitis (Wieckowska et al., 2008), have high serum levels of IL-6. Since all these (alcoholic liver disease, hepatitis B and C infections and steatohepatitis) conditions were the predisposing factors for hepatocarcinogenesis, hence establishing a bridge between IL-6 levels and hepatocarcinogenesis (Soresi et al., 2006). The tumour promoting effect of IL-6 has also been confirmed in an interesting study with IL-6 knockout mice, in which diethylnitrosamine (DEN) failed to induce tumours. Another remarkable outcome of this study was that the knockout of IL-6 abolished the gender differences in hepatocarcinogenesis. Generally, male wild type mice are more prone to tumor induction by DEN than females. However, IL-6 knockout mice both males and females did not develop liver tumours (Naugler et al., 2007).
8.6. Inducible nitric oxide synthase signalling

Inducible nitric oxide synthase (iNOS) produces sustained nitric oxide (NO) concentrations in response to pro-inflammatory agents. NO is a major mediator of chronic inflammation and may modulate tumorigenesis by regulating cell proliferation, survival, and migration, angiogenesis, drug resistance, and DNA repair (Lasagna et al., 2006; Hussain and Harris, 2007). In particular, iNOS might promote unrestrained cell growth via its ability to inactivate the retinoblastoma (pRb) pathway (Ying, et al., 2007). Some observations envisage a cross talk between iNOS and inhibitor of κB kinase (IKK)/nuclear factor-κB (NF-κB) and RAS/ERK pathways. The Ikk and NF-κB activities are strongly reduced in iNos knockout mice (Zingarelli et al., 2002). NO activates Ha-RAS/ERK pathway in T lymphocytes (Deora et al., 2000). Phosphorylated ERK activates iNos in melanoma (Ellerhorst et al., 2006) and NF-κB in HeLa cells (Zhao and Lee, 1999). The observation that the expression of iNOS and its downstream targets is highest in HCCs prone to progression both in rodents and humans, and that iNOS levels are directly correlated with genomic instability, proliferation rate and micro vessel density of HCC, and inversely correlated with apoptosis and patients’ survival (Calvisi et al., 2008), suggests that iNOS upregulation and changes in iNOS/NF-κB and iNOS/Ha-RAS/ERK cross-talks are prognostic markers for HCC. In conclusion, iNOS over expression contributes to growth deregulation in preneoplastic and neoplastic liver cells through a cross-talk with Ha-RAS/ERK and IKK-NF-κB axis.

9. Various signalling pathways in hepatocarcinogenesis

As discussed above, the key players in the progression from chronic liver diseases to hepatocarcinogenesis are inflammatory cytokines. Liver cells express various cytokine receptors including IL-1 (interleukin 1), TNF-α (tumour necrosis factor alpha) or IL-6 (interleukin 6)-receptors (Budhu and Wang, 2006).

9.1. TNF-α mediated signalling pathway in hepatocarcinogenesis

TNF-α is a member of the TNF / TNFR (tumour necrosis factor / TNF receptor) cytokine super family (Locksley et al., 2001) and is involved in various physiological and pathological processes including immunity, inflammation, tumour growth, transplant rejection or rheumatoid arthritis (Wang and Lin, 2008). TNF-α is synthesized as a type II transmembrane protein with an intracellular N-terminus arranged in stable homo-trimers. The cytokine exerts its signalling potential as membrane integrated protein and as soluble cytokine. Soluble TNF-α is released via proteolytic cleavage by the metalloprotease TNF-α.
converting enzyme (TACE) also known as ADAM 17 (A Disintegrin and Metalloprotease 17) (Figure 10) (Wajant et al., 2003).

There are two receptors for TNF-α viz, TNFR-1 and TNFR-2. TNFR-1 is ubiquitously expressed and constitutes the main receptor mediating the cellular effects of TNF-α. TNFR-2 is mainly expressed in haematopoietic cells and its biological role has not been completely elucidated yet (Balkwill, 2009). TNFR-1 is composed of a death domain consisting of an extracellular, transmembrane and an intracellular domain. It is the main receptor for the soluble form of TNF-α. TNFR-2 has no death domain and generally mediates the effects of the membrane bound precursor form of TNF-α. As their ligands, TNFRs can also be shed and act as soluble binding proteins, which reversibly bind soluble TNF-α for stabilising it and prolonging its half life (Balkwill, 2009; Wang and Lin, 2008). TNF-α is a pleiotropic cytokine that induces cellular responses including proliferation, production of inflammatory mediators and cell death (Wang and Lin, 2008). Upon binding to its most important receptor TNFR-1 leads to a conformational change followed by the binding of adapter molecules and the activation of multiple intracellular pathways (Hehlgans and Pfeffer, 2005). These adapter proteins including receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2) and Fas-associated death domain (FADD) interact with the TNFR-associated death domain (TRADD) and recruit key molecules important for the activation of either survival and proliferation pathways or apoptotic pathways (Wang and Lin, 2008).

At least four pathways are mediated upon binding of TNF-α to TNFR.

- A pro-apoptotic pathway induced by binding of caspase-8 to FADD,
- An anti-apoptotic one that is activated by the binding of cellular inhibitor of apoptosis protein-1 (cIAP) to TRAF-2,
- Apoptosis protein 1 activation which is mediated through TRAF-2 via JNK (c-Jun N-terminal kinases) signalling
- NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation by RIP.

Activation of the transcription factor NF-κB results in the expression of pro-proliferative and survival genes (Figure 10) (Szlosarek et al., 2006). The regulation of these pathways is of very importance because in many pathological conditions like cancer, there is an imbalance between apoptotic and survival signals (Balkwill, 2006).
TNF-α expression is very rare in healthy liver. However liver diseases are almost always associated with inflammation and therefore there is expression of TNF-α, which is induced in early stages of hepatic inflammation, triggering a cascade of other cytokines. It is also reported that high concentrations of TNF-α is found in patients suffering from hepatitis B or C virus infections (Nelson et al., 1997; Sheron et al., 1991) as well as from alcoholic and non alcoholic fatty liver disease (McClain et al., 2004; Kugelmas et al., 2003; Crespo et al., 2001). Moreover, in patients with hepatocarcinogenesis, serum levels of TNF-α were found to be increased correlating with disease severity and nutrition status (Wang et al., 2003). Whereas TNF-α induces cell death in injured livers (Bradham et al., 1998), the systemic administration of TNF-α promotes cell proliferation of healthy hepatocytes indicating that healthy livers have good defence mechanisms against TNF-α induced stress (Feingold et al., 1988). This pro-proliferative consequence of TNF-α was also observed after partial hepatectomy of the liver (Akermann et al, 1992; Yamada et al., 1997). So TNF-α mediates hepatocyte proliferation as well as hepatocyte cell death and is therefore a key regulator in the maintenance of liver homeostasis (Wullaert et al., 2007).

The role of TNF-α in the promotion phase of hepatocarcinogenesis was investigated in different animal models. Knight et al detected impaired pre-neoplastic changes in TNF-
receptor-1 knockout mice in which hepatocarcinogenesis has been induced by the administration of choline deficient and ethionine supplemented diet (Knight et al., 2000). In a very recent study rats treated with diethylnitrosamine (DEN) and were additionally exposed to a high fat diet leading to steatohepatitis (fatty liver disease characterised by liver inflammation with concurrent accumulation of fat in liver), showed a tumour promoting effect of steatohepatitis which was associated with elevated TNF-α/NF-κB-signalling (Wang et al., 2009). In another study, Mdr-2 knockout mice (a mouse model for inflammation related hepatocarcinogenesis) a tumour promoting effect of TNF-α via NF-κB signalling could be detected (Pikarsky et al., 2004). The results suggest that TNF-α activates a NF-κB dependent anti-apoptotic pathway leading to promotion of tumourigenesis. Furthermore TNF-α is involved in crucial steps of tumour development like tumour angiogenesis, invasion and metastasis (Wang and Lin, 2008).

9.2. The VEGF (Vascular Endothelial Growth Factor) signalling pathway

Vascular endothelial growth factor (VEGF) has been reported to be the main inducer of angiogenesis. VEGF, targeting vascular endothelial cells, is known to play an key role in liver regeneration, hepatic fibrogenesis, portal hypertension and hepatocarcinogenesis (Benjamin et al., 1999; Ferrara and Davis-Smyth, 1997). In addition to hypoxia, mutations in tumor suppressor genes and oncogenes, hepatocarcinogenesis are associated with the up-regulation of VEGF. Hepatocarcinoma is a strongly vascularized tumor and therefore depends directly on VEGF expression (Yamaguchi et al., 1998). During the process of hepatocarcinogenesis, expression of VEGF increases gradually from low grade dysplastic nodules to high grade dysplastic nodules and to early hepatocarcinogenesis (Park et al., 2000). The degree of VEGF expression during HCC development correlates with the vessel density (Park et al., 2000). High serum levels of VEGF significantly correlated with the presence of intra-hepatic metastasis, presence of microscopic venous invasion, advanced stage and post-operative recurrence. (Figure 11)
The activity of VEGF is mediated through three receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3. VEGFR-1 is expressed on endothelial cells and monocytes mediating cell motility. The proliferative and mitogenic activities of VEGF, as well as vascular permeability, are mediated primarily through VEGFR-2. VEGFR-3, also known as Flt-4, being homologous with the neuropilin-1 receptor, is thought to mediate lymphoangiogenesis.

9.3. The Interferon signalling pathway / JAK-STAT signalling pathway

Viral infections or any other chemical insult to the cell triggers the production of certain specific cytokines known as interferons (IFNs). Two types of IFNs exist, viz Type I and Type II. Type I IFNs are secreted in response to viral infections by various cell types, whereas, Type II IFN, also known as IFN-γ are involved in antigen specific immune response. It is produced by activated T-cells and macrophages upon mitogenic or antigenic stimulation of the immune defence machinery. In response to viral infections, the cell activates different signalling cascades in order to produce cytokines that both inhibit pathogen replication and stimulate immune responses (Akira et al., 2001; Huang et al., 2001). After specific phosphorylations, interferon regulatory factor 3 (IRF3) dimmers migrate into the nucleus to stimulate the expression of certain IFNs during the very early phase of the cellular response to infections, whereas IRF7 is recruited later for amplifying the interferon response. Interferon-stimulated genes encode for proteins displaying strong antiviral, anti-proliferative and anti-tumoral activity. However, the efficacy of IFN treatment in human tumors is hampered by rapid development of resistance. Viruses have developed different tactics to intercept IFN signalling, in order to inhibit IFN-induced...
antiviral responses (Katze et al., 2002). Many viruses perturb signalling components of the JAK-STAT pathway, thus preventing the proper cellular response to IFN.

Type I IFNs (only IFN-α and IFN-β are shown for simplicity), type II IFN (IFN-γ) and type III IFNs (IFN-λ1/ IL-29, IFN-λ2/IL-28A and IFN-λ3/IL-28B) bind to their receptor complex as in figure 12. All of them activate the canonical JAKSTAT pathway. Type III IFNs were reported to activate STAT1 and STAT2 through the activation of JAK kinases, leading to downstream gene induction in an ISGF3- or GAF/AAF-dependent manner. It is not yet clear which JAKs are associated with the type III receptor subunit (IFN-λR1). STAT5 is associated with Tyk2, while v-crk sarcoma virus CT10 oncogene homologue (avian)-like (CrkL) constitutively associates with the guanine-nucleotide-exchange factor (GEF) C3G. Following type I IFN stimulation, CrkL is recruited to Tyk2, which phosphorylates both STAT5 and CrkL, resulting in heterodimer formation. This dimer translocates into the nucleus to activate GAS-mediated gene induction. Type I IFNs also activate the PI3-kinase-mediated signalling pathway downstream of JAK1 and Tyk2, through the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2, most probably independently of STATs. The activated PI3-kinase consequently increases the activities of v-akt murine thymoma viral oncogene homologue 3(Akt) and protein kinase Cδ (PKCδ). Moreover, type I IFNs can activate MAP kinases, the so called extracellular signal-regulated kinase 2 (ERK2) and p38, both of which are capable of phosphorylating Ser 727 of STAT1 (Figure 12).
Figure 12. Activation of the JAK-STAT pathway and additional signalling pathways by three types of IFNs (Takaoka and Yanai, 2006).

9.4. The Wnt/β-catenin signalling pathway

The Wnt family consists of more than 15 closely related secreted glycoproteins. Receptors for the Wnt proteins are members of the frizzled family of transmembrane proteins, and the Wnt signal is transduced to a cytoplasmic protein, Dishevelled (Dvl). Upon activation by the Wnt signal, Dvl inhibits the activity of glycogen synthase kinase-3β (GSK-3β). (Figure 13) In the absence of the Wnt signal, GSK-3β phosphorylates β-catenin and induces the degradation of β-catenin. Thereby, the Wnt signal induces the accumulation of β-catenin, which in turn associates with TCF/LEF family transcription factors, altering the expression of Wnt signalling target genes (Akiyama, 2000).
The role of the Wnt/β-catenin pathway is well established in vertebrates in embryogenesis and hepatocarcinogenesis (Pennisi, 1998). The Wnt/β-catenin pathway emerged to be critical for biliary epithelial cell growth (Sekhon et al., 2004). It has been observed that, within first few minutes of partial hepatectomy, there is a significant increase in the total β-catenin protein. This was mediated by an epigenetic or post-translational mechanism and it occurred independently of transcriptional modifications (Monga et al., 2001). Approximately, 90-100% of hepatoblastomas have shown nuclear and cytoplasmic localization of β-catenin (Jeng et al., 2000). In addition, abnormal cytoplasmic and/or nuclear localization of β-catenin was observed in 30 - 46% of hepatic adenomas (Chen et al., 2002). Altered Wnt/β-catenin activation has been demonstrated in many cancers and it is one of the important aberrant pathways in hepatocarcinogenesis in man and animals (Laurent-Puig et al., 2001; Polakis, 2000). Moreover, more than 40% of HCV-associated hepatocarcinomas have been reported to display mutations in the β-catenin gene and nuclear accumulation of the respective protein (Huang et al., 1999) while HBV-related
hepatocarcinomas display an overall lower frequency of β-catenin mutations (Boutros et al., 1998).

9.5. The role of EGFR signalling system in hepatocarcinogenesis

The EGFR system mediates pro-survival and mitogenic signalling cascades; therefore dysregulated EGFR signalling may contribute to the progression from chronic liver disease to hepatocellular carcinoma. Ligands of EGFR including TGF-α, HB-EGF, EREG and AR are elevated in chronic liver diseases as well as in liver regeneration after partial hepaetectomy (Berasain et al., 2009). The situation is similar in HCC. Investigations of EGFR in human liver cancers indicated an over expression of the receptor in 68% of all cases correlating with metastasis and poor patient survival (Ito et al., 2001, Breuhahn et al., 2006). Moreover, TGF-α (AR) are up regulated in human HCC tissue samples (Sibilia et al., 2007). Different animal studies confirmed the essential role of EGFR and its ligands in liver regeneration, chronic liver disease and associated hepatocarcinogenesis. Thus hepatocyte proliferation is impaired in EGFR deficient livers (Natarajan et al., 2007) as well as in HB-EGF and AR knockout mice (Berasain et al., 2007).

Transgenic mice over-expressing TGF-α developed hepatocarcinogenesis and reacted with pronounced tumour development upon treatment with carcinogens (Sibilia et al., 2007). The main source of EGFR ligands in the liver is the mesenchymal compartment; so TGF-α, AR and HB-EGF are mostly produced by non parenchymal liver cells such as Kupffer cells or endothelial cells stimulating hepatocytes in a paracrine manner (Perugorria et al., 2008; Gressner, 1995; Drucker et al., 2006; Sagmeister et al., 2008).

9.6. MAP Kinase Pathway

Mitogen-activated protein kinases (MAPKs) are signalling components that are important in converting extracellular stimuli into a wide range of cellular responses. The intracellular mitogen-activated protein (MAP) kinase family were implicated in diverse cellular processes such as cell survival, differentiation, adhesion, and proliferation (Johnson and Nakamura, 2007; Chang et al., 2009). The ERK1 and ERK2 MAPKs are activated by mitogens and were found to be up regulated in human tumours, this has led to the development of inhibitors of this pathway for cancer therapeutics (Sebolt-Leopold and Herrera, 2004).

Two other major MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, which are also called stress activated protein kinase pathways, are also often deregulated in cancers. JNKs and p38 MAPKs are activated by environmental and
genotoxic stresses and have key roles in inflammation, as well as in tissue homeostasis, as they control cell proliferation, differentiation, survival and the migration of specific cell types (Nebreda and Porras, 2000; Weston and Davis, 2007; Rincon and Davis, 2009).

9.7. JNK signalling pathway

The JNK proteins are encoded by three genes, MAPK8 (which encodes JNK1), MAPK9 (which encodes JNK2) and MAPK10 (which encodes JNK3), which are alternatively spliced giving rise to at least ten isoforms (Gupta, et al., 1996). JNK1 and JNK2 are expressed in almost every cell, whereas JNK3 is mainly found in the brain (Cuevas et al., 2007; Bode and Dong, 2007). JNKS can be activated by the upstream MKK4 and MKK7 kinases. Although there are many JNK substrates, it is still a challenge to identify the molecular networks regulated by the individual JNK family members (Weston and Davis, 2007; Rincon and Davis, 2009). A major JNK target is the transcription factor AP1, which is composed of Fos and Jun family members (Eferl and Wagner, 2003). The oncogenic functions of JNKS are mostly based on their ability to phosphorylate JuN and to activate AP1, whereas their tumour suppressive functions are probably related to their pro-apoptotic activity.

The JNK/JuN pathway regulates a plethora of target genes that contain AP1-binding sites, including genes that control the cell cycle, as well as survival and apoptosis, metalloproteinases and nuclear hormone receptors, such as retinoid receptors (Altucci and Gronemeyer, 2001).

9.8. p38 MAPK signalling

There are four genes that encode p38 MAPKs: MAPK14 (which encodes p38α), MAPK11 (which encodes p38β), MAPK12 (which encodes p38γ) and MAPK13 (which encodes p38δ); two alternatively spliced isoforms of MAPK14 have also been reported (Lee et al., 1994; Sanz et al., 2000). The two major groups of proteins that are regulated by p38 MAPK-mediated phosphorylation are transcription factors, such as p53, activating transcription factor 2 (ATF2), Elk1, myocyte-specific enhancer factor 2 (MEF2) and C/EBPβ; and protein kinases, including MAPK-activated kinase 2 (MK2; also known as MAPK2), mitogen- and stress-activated protein kinase 1 (MsK1), MAP kinase-interacting serine/threonine kinase 1 (MNK1) and MNK2 (Figure 14). There is much evidence to support a role for p38α as a tumour suppressor, and this function of p38α is mostly mediated by both negative regulation of cell cycle progression and the induction of apoptosis, although the induction of terminal differentiation also contributes to its tumour
suppressive function (Dolado and Nebreda, 2008; Hui et al., 2007). However, p38α may also have oncogenic functions that are mediated by its involvement in key processes of cancer progression, such as invasion, inflammation and angiogenesis.

Figure 14: Activation of mitogen-activated protein kinase signalling pathways (Wagner and Nebreda, 2009)

10. Hepatocyte growth factor

Hepatocyte growth factor (HGF), also known as “scatter factor” represents a potent mitogen for hepatocytes carrying out its functions through binding to its receptor c-MET (HGFR) (Matsumoto and Nakamura, 1992). HGF is secreted by mesenchymal cells including stellate cells, myofibroblasts (Holt et al., 2009), Kupffer cells as well as endothelial cells (Nakamura, 1991) and acts on hepatocytes via paracrine mechanism. HGF is a potent growth factor for preneoplastic hepatocytes (Drucker et al., 2006) and enhanced DEN-
induced hepatocarcinogenesis (Horiguchi et al., 2002). The HGF-receptor - a receptor tyrosine kinase - is not only expressed in hepatocytes but also in endothelial cells (Huitfeldt et al., 1996) mediating important biological functions such as proliferation (Efimova et al., 2004), migration (Monvoisin et al., 1999), tissue regeneration (Borowiak et al., 2004) and angiogenesis (You and McDonald, 2008).

Expression of HGF and its receptor in hepatocellular carcinoma is rather controversial, as several studies have demonstrated that there are high serum and tissue HGF and c-MET levels in patients with chronic liver diseases such as chronic hepatitis (Noguchi et al., 1996; Barreiros et al., 2009). In contrast HGF-levels in HCC samples were very low or could not be detected at all (Noguchi et al., 1996; Selden et al., 1994). Although the exact role of HGF in hepatocarcinogenesis needs to be further investigated, its involvement in hepatocarcinogenesis is beyond all questions; as its mitogenic potential was demonstrated in numerous liver regeneration models (Fausto et al., 2006).

10.1. Growth factors and hepatocarcinogenesis

Chronic inflammatory liver diseases and hepatocarcinogenesis are not only characterized by activation of inflammatory cytokines, but also by elevated growth factor levels reflecting the response of the liver to inflammation and injury with the purpose of liver regeneration and repair (Fausto et al., 2006). However dysregulated growth factor signalling favours growth of preneoplastic hepatocytes and can contribute to development of hepatocarcinogenesis (Breuhahn et al., 2006; Drucker et al., 2006; Sagmeister et al., 2008). A number of growth factor signalling pathways including the insulin-like growth factor (IGF), hepatocyte growth factor (HGF), wingless (Wnt), transforming growth factor beta (TGF-β), heparin binding epidermal growth factor like growth factor (HB-EGF) and the epidermal growth factor receptor (EGFR) signalling pathways were altered in inflammation and associated hepatocarcinogenesis (Breuhahn et al., 2006).

10.2. The ErbB family of receptors and their ligands

ErbB receptors and their ligands (the EGF family of growth factors) are involved in proliferation, motility, differentiation and survival of various cell types. In addition to their physiological roles they are important factors in the development and progression of tumours (Miyamoto et al., 2006). ErbB receptors belong to the tyrosine kinase family and comprise four members viz, EGFR (ErbB1, HER1), ErbB2 (HER2, neu), ErbB3 (HER3) and ErbB4 (HER4) (Iwamoto and Mekada, 2006). These receptors consist of an extracellular ligand binding domain, a single hydrophobic transmembrane domain and a
conserved cytoplasmic tyrosine kinase-containing domain (Bazley and Gullick, 2005) (Figure 15), although the tyrosine kinase domain of ErbB3 contains additional amino acids and therefore has no kinase activity (Guy et al., 1994).

Since the kinase domain is highly conserved, the extracellular domains of the receptors exhibit different specificities in ligand binding. Ligands of ErbB receptors are different growth factors of the EGF family - epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin (AR), heparin binding growth factor like growth factor (HB-EGF), betacellulin, epiregulin (EREG), epigen, neuregulin-1 (NRG-1), NRG-2, NRG-3, NRG-4, NRG-5 (tomoregulin), NRG-6 (epiglycan C) (Higashiyama et al., 2008; Bazley and Gullick, 2005). They are classified into three groups due to their specificity in binding to the different ErbB receptors; ErbB2 has no ligands (Table 1). All these growth factors have an EGF-like domain composed of three disulfide-bonded intra-molecular groups conferring binding specificity (Harris et al., 2003).
ErbB Receptors

<table>
<thead>
<tr>
<th>Ligands</th>
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<th>ErbB2</th>
<th>ErbB3</th>
<th>ErbB4</th>
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<tr>
<td>EGF</td>
<td>-</td>
<td>NRG-1</td>
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<td>TGFz</td>
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<td>NRG-2</td>
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<td>Amphiregulin</td>
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<td>Epiregulin</td>
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<td>betacellulin</td>
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<td>HB-EGF</td>
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<td>Epigen</td>
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<td>HB-EGF</td>
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Table: ErbB receptors and their ligands (Normanno et al., 2006)

Binding of the soluble growth factors to ErbB induces homo- or hetero-dimerization of receptors and auto-phosphorylation of the cytoplasmic tyrosine kinase domain resulting in induction of various signalling cascades like the ras/raf/MEK/MAPK pathway or PI3K (Phosphatidylinositol-3-kinase)-pathway as well as in the activation of various transcription factors (Sebastian et al., 2006; Normanno et al., 2006). ErbB2 has no ligands but have an important role in hetero-dimerization with other activated ErbB receptors (Graus-Porta et al., 1997)

11. Epithelial mesenchymal interactions

11.1. Epithelial mesenchymal interactions in early hepatocarcinogenesis

The coupling between the epithelial and the mesenchymal compartment plays an essential role in the multi step process of hepatocarcinogenesis. Vast number of cytokines, growth factors and ROS were generated by mesenchymal cells, which contribute to the development of hepatocarcinogenesis. To study epithelial - mesenchymal interactions in early hepatocarcinogenesis a unique model has been developed, in which rats are treated with the genotoxic substance N-nitrosomorpholine (NNM), which induces initiated hepatocytes expressing the marker placental glutathione-S-transferase (GSTp). GSTp-positive (GSTp-pos) cells show generally a higher rate of DNA-replication than GSTp-negative (GSTp-neg) ones indicating an inherent growth advantage of the initiated/premalignant cell population (Löw-Baselli et al., 2000). In order to investigate the role of mesenchymal cells (MC) in this system, two preparations of hepatocytes were made, whereas the first preparation contained approximately 5% of mesenchymal cells, the
second one was purified from them. It was observed, that DNA-synthesis of initiated hepatocytes increased significantly in the presence of mesenchymal cells. This effect could be further enhanced by lipopolysaccharide stimulation of mesenchymal cells (Drucker et al., 2006). It was assumed that pro-inflammatory cytokines and growth factors from the mesenchymal compartment are involved in the outgrowth of premalignant hepatocytes and found that HB-EGF is highly up regulated in endothelial cells and Kupffer cells by various inflammatory stimuli and increases cell replication of initiated hepatocytes (Sagmeister et al., 2008). These results advocate that interaction of mesenchymal cells with the epithelial compartment plays an important role in early hepatocarcinogenesis.

11.2. Epithelial mesenchymal interactions in late hepatocarcinogenesis

During the last century, there is an enormous progress in cancer research in identifying the molecular mechanisms in carcinogenesis; as carcinogenesis was considered as a multi step process with accumulation of numerous genetic and epigenetic alterations in cancer cells. The consequence was the detection, characterization of many oncogenes and tumour suppressor genes as well as fundamental proceedings in the development of new therapeutic strategies. However, most of the studies were focused on the epithelial compartment and neglected the heterogenous and complex nature of carcinomas, which consists not only of cancer cells derived from epithelial cells, but also of a surrounding mesenchymal tissue- the tumour stroma or tumour microenvironment (Figure 16) (Hanahan and Weinberg, 2000).

![The Reductionist View](image1)

![A Heterotypic Cell Biology](image2)

**Figure 16:** Tumours represent heterogenous tissues (Hanahan and Weinberg, 2000).

The connective tissue or the stroma of the tumour and is built up by the tumour matrix - a specific type of extracellular matrix (ECM), and by cellular components involving fibroblasts, immune cells like tumour associated macrophages or lymphocytes and
endothelial cells. This conglomerate of different cell types and ECM-macromolecules has been recognized as an important mediator in carcinogenesis via supporting the survival and proliferation of epithelial cells in a paracrine manner (Mueller and Fusenig, 2004; Albini and Sporn, 2007). Tumour cells have the capability to modulate its stroma in order to create a supportive environment for tumour generation. They activate their stromal environment via secretion of stroma-modulating growth factors and inflammatory cytokines. These factors induce stromal reactions such as angiogenesis and inflammatory response as well as cause a disturbance of tissue homeostasis resembling the process of wound healing (Mueller and Fusenig, 2004). Dvorak described tumours as “wounds that do not heal” demonstrating the crucial role of inflammatory processes in tumorigenesis and the participation of the tumour stroma in these processes (Dvorak, 1986). In the process of hepatocarcinogenesis, inflammation plays a determining role in a two-fold way. On the one hand chronic inflammation is an important predisposing factor for the development of hepatocellular carcinoma (Coussens and Werb, 2002), on the other hand inflammatory cells are components of the tumour microenvironment arising independent of chronic inflammation (Bhowmick and Moses, 2005). Whereas normal physiological inflammatory processes result in tissue remodelling and repair, however hepatocarcinogenesis is characterized by sustained cycles of necrosis-inflammation-regeneration, which is an attempt to reestablish homeostasis, but instead leads to promotion of tumour growth (Albini and Sporn, 2007; Farazi and DePinho, 2006).

12. Role of p53 in Hepatocarcinogenesis

p53, the “guardian of the genome,” is a sequence-specific transcription factor and its activity is regulated via strong control of P53 protein levels. Normally, P53 levels are kept low by associating it with the mdm2 oncogene product, which binds p53 and shuttles it out of the nucleus for proteolytic degradation. Regulation of p53 levels is done by two checkpoint pathways that are activated in response to DNA damage or oncogene-induced cell proliferation (Fig. 17-1). The loss of P53 function revokes these checkpoints and helps tumor cells to escape cell cycle arrest, senescence, or apoptosis despite accumulation of mutations and unusual passage through the cell cycle.

Acquired mutation in p53 is the most common genetic alteration found in human cancer (>50%); the Li-Fraumeni familial cancer syndrome is caused by germ line mutation, a genetic lesion. In various tumors, one of the p53 allele on chromosome 17p is deleted and the other one is mutated. The mutations often revoke the DNA binding function of p53 required for its activity as a transcription factor and its function as a tumor-suppressor, and
also results in high intracellular levels of p53 protein. When the p53 pathway does not get activated, it compromises cell cycle arrest, attenuates apoptosis induced by DNA damage or other stimuli, and predisposes cells to chromosome instability. This genomic instability largely increases the probability that p53 null cells will acquire additional mutations and become malignant. In a nutshell, it is likely that all human cancers have genetic alterations that inactivate the Rb and p53 tumor-suppressor pathways.

Tumors showing mutant p53 are more resistant to radiation therapy and chemotherapy than tumors with wild-type p53. If the transcriptional functions of the mutant p53 could be re-established in tumor cells, it might result in massive apoptosis, while normal cells would be protected because they show very low levels of wild type p53. Investigators have screened chemical libraries for compounds that inhibit tumor cell growth in a mutant p53-dependent manner. One compound entered cells and actuated mutant p53 to adopt an active conformation such that p53-dependent transcriptional activation was restored and apoptosis was selectively induced.

This compound also had anti-tumor activity in murine xenograft models. Other investigators have recognised a low-molecular-weight, cell-permeable compound that inhibits the apoptotic functions of wild-type p53 found in normal host cells. This compound protected mice from the toxic effects of radiation therapy and chemotherapy, including bone marrow suppression, gastrointestinal dysfunction, and hair loss. Collectively, these approaches provide proof of principle for the pharmacologic manipulation of p53 function (mutant or wild-type) that could largely enhance therapeutic efficacy simultaneously decreasing toxicity (Figure 17). (Vousden and Lane, 2007).

Figure 17: Induction of p53 by the DNA damage and oncogene checkpoints. (Robert and Longo: Cancer cell biology and angiogenesis)
Previous knowledge of the various molecular events that govern the cell cycle regulation have led to the development of viruses that replicate selectively in tumor cells with precise genetic lesions. Such “oncolytic” viruses include adenoviruses structured to replicate in tumor cells that do not have functional p53 or have flaws in the pRB pathway. The former group includes an adenovirus mutant which had a deleted viral p55 protein (which binds and inhibits p53); this virus selectively replicates in tumor cells lacking p53 function. This virus was efficient in phase II clinical trials of head and neck tumors, especially in combination with 5-fluorouracil and cisplatin (50% partial or complete response). The complexities of virus-host interactions (i.e., immune response against replicating virus) will require further improvements of this new technology before this approach can be completely utilised clinically.

13. Advanced Treatment of Liver Disease

Liver transplantation is usually the best option for the treatment of liver cancer or cirrhosis. However, there is an extreme scarcity in the availability of donor organs, and there are restrictions on who can receive those liver transplants. Because of these problems, researchers constantly sought for alternatives. Some of the important areas of research involve gene therapy, xenotransplants, and bioartificial livers.

1) Scientists have found that the amount of fibrosis that occurs in damaged livers can be reduced and even reversed by controlling the level of a gene named HGF (hepatocyte growth factor) in rats. If this can be extended to humans, it would surely have enormous advantages.

2) Xenotransplants are being aimed at for all types of organs replacements. Various companies have developed breeds of transgenic animals that won’t present as an immunological barrier like the xenotransplants used in the past. For example, Ximerex, Inc is developing hybrid livers that consist of pig livers partially repopulated with human cells. Baboon livers have already been transplanted into humans, although with a poor success rate so far.

3) Bioartificial livers are also being developed. Nowadays, they are used more often for the time being, while waiting for a liver transplant. For example, the HepatAssist Liver Support System employs a hollow-fiber membrane bioreactor containing $7 \times 10^8$ cryopreserved porcine hepatocytes along with associated equipment to make the liver function temporarily. In one study using the HepatAssist system (which is now in phase III clinical trials), 30 day patient survival rates improved to 90% compared to a normal level of 50-60%.
It can be concluded that the mesenchymal compartment plays a crucial role in the multistep process of hepatocarcinogenesis. Moreover, it is most likely that the mesenchymal cells contribute to the progression from hepatic inflammation to HCC via release of many cytokines, growth factors as well as ROS/RNS which together support neoplastic transformation and proliferation of (pre)neoplastic hepatocytes. However, this aspect has not been delineated in detail so far. The following part reviews the knowledge available on the role of mesenchymal liver cells in liver physiology and pathology.

14. Apoptosis and its association with cancer

Apoptosis, an evolutionary conserved genetic program of cell death in higher eukaryotes, is a basic process involved in cellular development and differentiation (Danial and Korsmeyer, 2004; Green and Evan, 2002). Apoptosis may be essential for the prevention of tumor formation, and its deregulation is widely believed to be involved in pathogenesis of many diseases, including cancer (Thompson, 1995). In almost all instances, deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression (Figure 18). (Evan and Vousden, 2001).

Apoptosis involves a “cleaner” type of cell death, in which the chromatin is condensed; the DNA becomes fragmented forming vesicles known as “apoptotic bodies”. These are rapidly phagocytosed by the macrophages with the result that the cell disappears without any inflammatory phenomena (Lauber et al., 2004). Apoptosis induction might be achieved in several ways, for example, by promoting the expression of pro-apoptotic factors while reducing the expression of anti-apoptotic factors only in the tumour cells (Ozben, 2007).

Apoptosis can be initiated by three different pathways:

1. The extrinsic pathway, which can be triggered by ligation of death receptors and subsequent caspase 8 activation.
2. The intrinsic pathway, initiated by internal cellular stress followed by activation of caspase 9.
3. The granzyme B pathway, where the cytotoxic cell protease granzyme B is delivered to sensitive target cells.

Each of these pathways congregate to a common execution phase of apoptosis that requires proteolytic activation of caspases 3 and/or 7 from their inactive zymogens (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Biochemically, the main features of apoptosis include caspase cascade activation and DNA fragmentation (Adams, 2003). Mitochondria also play a key role in mediating apoptosis induced by diverse stimuli.
They release pro-apoptotic proteins (cytochrome c, Smac, Omi, AIF, and EndoG) into the cytosol and their release is regulated by proteins belonging to the Bcl2 family. Apoptosis pathways can be initiated via different stimuli that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway).

14.1. Extrinsic Apoptosis Pathway

The extrinsic apoptotic pathway triggering receptors are located in the cell membrane and are activated by extracellular ligands. Typical death receptors are Fas (fibroblast associated antigen, also called Apo-1 or CD95) and tumour necrosis factor receptor (TNF-R) 1; they belong to TNF-R family and contain a cytosolic death domain. Ligation of death receptor causes formation of death inducing signalling complex (DISC) (Kischkel et al., 1995; Ashkenazi and Dixit, 1998), in which the adaptor proteins FADD and/or TRADD bind with their death domain in the cytoplasmic region of the receptors (Boldin et al., 1995). The receptor induced pathway leads to the recruitment of caspase 8 or 10 (initiator caspases) to the DISC (Bodmer et al., 2000). The activated caspase then proteolytically activates downstream effector or executioner caspases that degrade cellular targets. Activated caspase 8 then directly cleaves pro-caspase 3 or other executioner caspases, eventually leading to the apoptosis. Caspase 8 can also cleave the -only BH3 protein Bid. The resulting truncated Bid (tBid) then moves to the mitochondria and induces cytochrome c release, leading to activation of caspase 9 and caspase 3.
Figure 18: The molecular mechanisms of apoptosis (Ghavami et al., 2009).

14.2. The intrinsic mitochondrial pathway

The intrinsic pathway is initiated within the cell and it can be triggered by variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca²⁺, severe oxidative stress, the actions of some oncoproteins and tumour suppressor genes (p53), viral virulence factors, and most chemotherapeutic agents (Karp, 2008). Regardless of the stimuli, this pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm (Danial and Korsmeyer, 2004). This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family, named after the BCL2 gene originally observed at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma (Tsujimoto et al., 1984). There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Reed, 1997). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the
pro-apoptotic proteins act by promoting the release of cytochrome-c. The balance between the pro- and anti-apoptotic proteins determines whether apoptosis would be initiated or not (Reed, 1997). Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct inhibitor of apoptosis proteins (IAP) Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Kroemer et al., 2007). Cytoplasmic release of cytochrome c activates caspase 3 via the formation of a complex known as apoptosome which is composed of cytochrome c, Apaf-1 and caspase 9 (Kroemer et al., 2007). On the other hand, Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to IAPs which subsequently leads to disruption in the interaction of IAPs with caspase 3 or 9 (Kroemer et al., 2007; LaCasse et al., 2008).

14.3. The common pathway
The execution phase of apoptosis involves the activation of a series of caspases. The upstream caspase for the intrinsic pathway is caspase 9 while that of the extrinsic pathway is caspase 8. The intrinsic and extrinsic pathways converge to caspase 3. Caspase 3 then cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis (Ghobrial et al., 2005). In addition, downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonuclease family. They also have an effect on the cytoskeleton, cell cycle and signalling pathways, which together contribute to the typical morphological changes in apoptosis (Ghobrial et al., 2005).

Cancer can be viewed as the result of a succession of genetic changes during which a normal cell is transformed into a malignant one while evasion of cell death is one of the essential changes in a cell that cause this malignant transformation (Hanahan and Weinberg, 2000). As early as the 1970's, Kerr et al had linked apoptosis to the elimination of potentially malignant cells, hyperplasia and tumour progression (Kerr et al., 1972). Hence, reduced apoptosis or its resistance plays a vital role in carcinogenesis. There are many ways by which a malignant cell can acquire reduction in apoptosis or apoptosis resistance. Generally, the mechanisms by which evasion of apoptosis occurs:

1. Disrupted balance of pro-apoptotic and anti-apoptotic proteins.
2. Reduced caspase function and
3. Impaired death receptor signalling.
15. **Role of Oxidative stress in hepatocarcinogenesis**

One of the main driving force, which helps to sustain human life, are the biochemical reactions which take place within the organelles and cells of the body. These natural biochemical processes occurring in cells lead to the generation of reactive oxygen species (ROS). It has been established that reactive oxygen species can be both harmful and beneficial in biological systems depending on the environment within the cell (Lopaczynski and Zeisel, 2001; Glade 2003). Beneficial effects of ROS involve the physiological roles in cellular responses to noxia such as defence against infectious agents, and in the function of a number of cellular signalling systems. In contrast, at high concentrations, ROS can mediate damage to cell structures, including lipids and membranes, proteins and nucleic acids; this damage is often referred as “oxidative stress” (Poli *et al.*, 2004). The harmful effects of ROS are balanced by the action of antioxidants, some of which are enzymes present in the body (Halliwell, 1996). Despite the presence of the cell’s antioxidant defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been implicated in aging and age-dependent diseases such as...
cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions (Rahman, 2003)

Figure 20: Reactive oxygen species (ROS) and their role in carcinogenesis (Klaunig et al., 2010)

Oxidative stress is often marked as a key link between chronic inflammation and the development of hepatocarcinogenesis through dysregulated cytokine and growth factor signalling in inflamed livers. Oxidative stress results due to overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), exceeding antioxidant capacity. ROS and RNS interact with various intracellular molecules such as proteins, DNA as well as lipids causing modification of proteins, oxidative DNA damage and lipid peroxidation. All these effects are implicated in the multi step process of hepatocarcinogenesis (Sasaki, 2006). The major source of ROS in inflamed liver is the mesenchymal cells including Kupffer cells and neutrophils. Moreover, the activity of membrane bound NADPH oxidase in activated Kupffer cells facilitates production of superoxide, which are involved in tumour initiation and promotion (Teufelhofer et al., 2005; Roberts et al., 2007). The inflammatory neutrophils also generate numerous oxidants such as superoxide anion, hydrogen peroxide or the potent DNA reactive molecule hydroxyl radical (Sasaki, 2006).
Oxidative stress has also been reported to be characteristic during Hepatitis viral infections. Elevated serum and tissue markers of lipid peroxidation as well as oxidative DNA damage have been observed in specimens of patients suffering from HBV and HCV infections (Sumida et al., 2000; Mahmood et al., 2004; Fujita et al., 2008). Oxidative stress in hepatitis may be associated with chronic inflammation characterized by the sustained generation of ROS by Kupffer cells and neutrophils; also virus associated proteins may be involved in the generation of ROS (Sasaki, 2006). With regard to non-alcoholic fatty liver diseases (NAFLD), the involvement of oxidative stress in the progression from steatosis via steatohepatitis to hepatocarcinoma seems to be likely. NAFLD is characterized by increased lipid peroxidation rates due to high amounts of ROS and free fatty acids (FFA) (Qian and Fan, 2005). ROS as well as lipid peroxidation end products such as 4-hydroxy-2-nonenal (HNE) can interact with DNA resulting in modified nucleic acids and/or DNA adducts. Increased hepatic expression of HNE-adducts and of 8-hydroxydeoxyguanosine has been observed in specimens from NAFLD patients (Seki et al., 2002). These promutagenic lesions are further fixed to mutations by enhanced proliferation before DNA repair enzymes can remove the damage.

Induction of oxidative stress and DNA damage results in either the direct induction of liver cancer (initiation) or contributes indirectly to the cancer process (Tumour Promotion). Hydroxylation of DNA and the subsequent production of initiated cells by ROS have been suggested for the initiation step in carcinogenesis. Hadler et al., has suggested that mitochondrial mutations may lead to a modified growth advantage in initiated cells (Hadler et al., 1971). Promotion occurs when initiated hepatocytes are resistant to the toxic effects, and xenobiotics gave them a selective growth advantage over normal cells. Cell proliferation or inhibition of apoptosis in initiated cells has also been proposed as a mechanism of ROS generated carcinogenesis by xenobiotics (Solt and Farber, 1977). DEN, a ROS generating carcinogen (Nakae et al., 1997) induces many preneoplastic lesions while N-ethyl-N-nitrosourea (ENU), a non-ROS generating carcinogen, induces less preneoplastic lesions in the rat liver (Sanchez-Perez et al., 2005). Nakae et al., showed that initiation with DEN induced liver DNA-8-hydroxy deoxygaunosine adducts and suggested that oxidative stress participate in hepatocarcinoigenesis (Nakae et al., 1997). 8-hydroxy-gaunosine is oxidative DNA damage marker and is induced by various hepatocarcinogens during hepatocarcinogenesis. For example: during choline deficient diet and after administration of cuprofibrat, one of the more efficient peroxisome proliferators that induce liver cancer in the rats (Floyd et al.,
Various chemicals such as thioacetamide, bromobenzene, carbon tetrachloride, ethanol, paraquat, menadione etc, undergo redox cycling and activate oxygen by reduction play important role in the toxicity of these chemicals (Tribble et al., 1987). Redox cycling is also involved in the toxicity of many hydroquinones, quinones, metal chelates, nitro compounds, amines and azo compounds (Kappus and Sies, 1981). ENU, a potent monofunctional ethylating and mutagenic agent in a variety of systems, its hepatocarcinogenic effect require previous oxidative stress conditions, such as partial heptectomy (PH) or phenobarbital treatment. Both PH and phenobarbital treatment generally generate ROS and increase the mutagenicity and carcinogenicity induced by ENU. Oxidative stress induced expression of immediate early genes such as c-myc, c-jun and c-fos and stimulation of transcription factors NF κB and AP-1, which can lead to enhanced cell proliferation. Evidence are shown with U937 cell treated with two well known nitrosamines, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone and diethylnitrosamine showed the generation of ROS; these activate nuclear factor kappa B (NF-κB); subsequently, cyclooxygenase-1 (COX-1) activity, and this pathway increases prostaglandin E2 (PGE2) synthesis and these events have a probable role in inflammation and cancer.

Stimulation of DNA is critical to all stages of cancer. Before the formation of preneoplastic lesions, enhanced DNA synthesis is a necessary event, which allows the fixation of spontaneously occurring mutations or mutations resulting from the genotoxic compounds. Oxidative stress cause an increase in DNA synthesis through a variety of mechanisms such as, activation of PKC activity and oncogenes such as, AP-1 and NF-κB and release of calcium ions from cellular stores which may in turn lead to mitotic events. Various hepatocarcinogens such as dieldrin, CCl₄, thioacetamide, 2-AAF, barbiturates, organochlorines and metals have been shown to produce an increase in the ROS in the liver. Several known genotoxic compounds and oxidative stress has also been shown to inhibit gap intracellular communication and protein kinase C (PKC) induction, which can lead to enhance hepatocellular proliferation. During hepatocarcinogenesis, participation of oxidative stress depends on both direct alkylating DNA damage and concomitant alterations of signalization produced by ROS.

16. Antioxidant defence system

The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans beings have evolved with a highly complex antioxidant systems (enzymatic and non-enzymatic), which work synergistically, and in
combination with each other to protect the cells and organ systems of the body against free radical damage. The antioxidants can be endogenous or obtained exogenously e.g., as a part of a diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants.

An ideal antioxidant should have following properties:

- It should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels.
- It should also work in both aqueous and/or membrane domains and effect gene expression in a positive way.

Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well being. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions.

The most efficient enzymatic antioxidants involve glutathione peroxidase, catalase and superoxide dismutase (Mates et al., 1999). Nonenzymatic antioxidants include Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds (McCall and Frei, 1999). Some antioxidants can interact with other antioxidants regenerating their original properties; this mechanism is often referred to as the “antioxidant network” (Sies et al., 2005). There is growing evidence to support a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in diseases associated with aging including cancer.

16.1. Enzymatic antioxidants

16.1.1. Glutathione peroxidase

There are two forms of this enzyme, one which is selenium-dependent (GPx) and the other, which is selenium-independent (glutathione-S-transferase, GST) (Mates et al., 1999). The differences are due to the number of subunits, catalytic mechanism, and the bonding of selenium at the active centre, and glutathione metabolism is one of the most important antioxidative defence mechanisms present in the cells. There are four different Se-dependent glutathione peroxidases present in humans (Chaudière and Ferrari-Iliou, 1999), and these are known to add two electrons to reduce peroxides by forming seleno-enzymes allow them to eliminate...
peroxides as potential substrates for the Fenton reaction. Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress (Chaudière and Ferrari-Iliou, 1999).

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

16.1.2. Catalase
This enzyme is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Mates et al., 1999).

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

16.1.3. Superoxide dismutase (SOD)
This is one of the most effective intracellular enzymatic antioxidants and it catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide. Superoxide dismutase exists in several isoforms, which differ in the nature of active metal centre, amino acid composition, co-factors and other features. There are three forms of SOD present in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extra cellular-SOD (Landis and Tower 2005). Superoxide dismutase neutralizes superoxide ions by going through successive oxidative and reductive cycles of transition metal ions at its active site (Chaudière and Ferrari-Iliou 1999). Cu, Zn-SOD has two identical subunits with a molecular weight of 32 kDa (Mates et al., 1999) and each of the subunit contains as the active site, a dinuclear metal cluster constituted by copper and zinc ions, and it specifically catalyzes the dismutation of the superoxide anion to oxygen and water. The mitochondrial Mn-SOD is a homotetramer with a molecular weight of 96 kDa and contains one manganese atom per subunit (Mates et al., 1999), and it cycles from Mn(III) to Mn(II), and back to Mn(III) during the two-step dismutation of superoxide. Extra cellular superoxide dismutase contains copper and zinc, and is a tetrameric secretory glycoprotein having a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate (Mates et al., 1999), however, its regulation in mammalian tissues occurs primarily in a manner coordinated by cytokines, rather than as a response to oxidative stress.
16.1.4. Xanthine oxidase (XO)
Xanthine oxidase is a complex enzyme containing flavins, molybdenum, iron, sulphide cofactors. It catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. It is present in ample amounts in liver and jejunum.

\[
\begin{align*}
2O_2^{-} + 2H^+ & \rightarrow H_2O_2 + O_2 \\
\text{Hypoxanthine} + O_2 & \rightarrow \text{Xanthine} + H_2O_2 \\
\text{Xanthine} + O_2 + 2H_2O & \rightarrow \text{Uric acid} + 2H_2O_2
\end{align*}
\]

16.1.5. Quinone reductase (QR)
Quinone reductase prevent the single electron reduction of quinones to semiquinone free radical intermediates be catalyzing its two electron reduction to hydroquinones or undergoes conjugation to form glucuronate or sulphate, which are stable and eliminated from quinone. Quinone reductase is also known as diaphorase. This enzyme system provides a protective mechanism by passing the quinone redox cycle (Prochaska et al., 1985)

16.2. Non-enzymatic antioxidants
16.2.1. Vitamin E or α-tocopherol
This is a fat-soluble vitamin existing in eight different forms. In humans, α-tocopherol is the most active form, and is the major powerful membrane bound antioxidant employed by the cell (Hensley et al., 2004). The main function of Vitamin E is to protect against lipid peroxidation (Pryor, 2000), and there is also evidence to suggest that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical, and the α-tocopherol radical can therefore be reduced to the original α-tocopherol form by ascorbic acid (Kojo, 2004).

16.2.2. Vitamin C (ascorbic acid)
This is an important and powerful water-soluble antioxidant and thus works in aqueous environments of the body. Its primary antioxidant partners are Vitamin E and the carotenoids as well as working along with the antioxidant enzymes. Vitamin C cooperates with Vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (Carr and Frei, 1999; Kojo, 2004), and also raises intracellular glutathione
levels thus playing an important role in protein thiol group protection against oxidation (Naziroglu and Butterworth 2005).

16.2.3. Thiol antioxidants
The major thiol antioxidant is the tripeptide glutathione (GSH), which is a multifunctional intracellular antioxidant and is considered to be the major thiol-disulphide redox buffer of the cell (Masella et al., 2005). It is abundant in cytosol, nuclei, and mitochondria, and is the major soluble antioxidant in these cell compartments (Masella et al., 2005). Glutathione has also been shown to play a role in cell senescence since studies involving human fibroblasts have shown that the intracellular glutathione level has a strong influence on the induction of a post-mitotic phenotype, and that by implication depletion of glutathione may play a significant role in the cellular aging in human skin (Alaluf et al., 2000). The reduced form of glutathione is GSH, glutathione, whilst the oxidized form is GSSG, glutathione disulphide. The antioxidant capacity of thiol compounds is due to the sulphur atom, which can easily accommodate the loss of a single electron (Karoui et al., 1996). Oxidized glutathione (GSSG) is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Dröge, 2002). The main protective roles of glutathione against oxidative stress are that it can act as a co-factor for several detoxifying enzymes, participate in amino acid transport across plasma membrane, scavenge hydroxyl radical and singlet oxygen directly, and regenerate Vitamins C and E back to their active forms (Masella et al., 2005).

16.2.4. Thioredoxin
Another thiol antioxidant is the thioredoxin (TRX) system; these are proteins with oxidoreductase activity and are ubiquitous in both mammalian and prokaryotic cells (Holmgren, 1985). It also contains a disulphide and possesses two redox-active cysteins within a conserved active site (Cys-Gly-Pro-Cys) (Nakamura et al., 1997). Thioredoxin contains two adjacent -SH groups in its reduced form that are converted to a disulphide unit in oxidized TRX when it undergoes redox reactions with multiple proteins. Thioredoxin levels are much less than GSH, however, TRX and GSH may have overlapping as well as compartmentalized functions in the activation and regulation of transcription factors (Valko et al., 2006).

16.2.5. α-Lipoic acid (ALA)
The third important thiol antioxidant is the natural compound α-Lipoic acid (ALA), which is a disulphide derivative of octanoic acid and is sometimes referred to as thiothic acid. It is
both water and fat soluble, and therefore, is widely distributed in both cellular membranes and the cytosol of eukaryotic and prokaryotic cells. α-Lipoic acid is readily absorbed from the diet and is converted rapidly to its reduced form, dihydrolipoic acid (DHLA) (Smith et al., 2004). Both ALA and DHLA are powerful antioxidants and they exert their effects by scavenging free radicals, metal ion chelation and antioxidant recycling, and repairing protein damage due to oxidative stress either in the cytosol or hydrophobic domains (Navari-Izzo et al., 2002). Dihydrolipoic acid is a stronger antioxidant than lipoic acid and can act synergistically with other antioxidants such as glutathione, ascorbate and tocopherol. However, it can also exert pro-oxidant properties both by its iron-reducing ability and by its ability to generate sulfur-containing radicals that can damage proteins (Navari-Izzo et al., 2002).

16.3. Plant antioxidants

16.3.1. Carotenoids

These are mainly coloured pigments present in plants and microorganisms and epidemiological studies have revealed that an increased consumption of a diet rich in carotenoids is correlated with a lower risk of stress related diseases. Carotenoids contain conjugated double bonds and their antioxidant activity arises due to the ability of these to delocalize unpaired electrons (Mortensen et al., 2001). This is also responsible for the ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity of carotenoids with free radicals. The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule, which determines their lowest triplet energy level. They can also scavenge peroxy radical thus preventing damage in lipophilic compartments (Stahl and Sies, 2003), however, the carotenoid β-carotene can also act as a pro-oxidant causing an increase in lipid peroxidation (Palozza et al., 2003). The concentrations of carotenoids and the partial pressure of oxygen are also important factors in their effectiveness as antioxidants. Carotenoids, in particular β-carotene exhibit antioxidant properties at low oxygen partial pressure but become pro-oxidants at high pressures of oxygen and similarly, at high carotenoid concentrations, pro-oxidant behaviour is displayed (Stahl and Sies, 2003).

16.3.2. Flavonoids

These are a broad class of low molecular ubiquitous groups of plant metabolites and are integral part of the human diet (Rice-Evans 2001). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings and during metabolism hydroxyl groups
are added, methylated, sulfated or glucuronidated. There is intense interest in flavonoids due to their anti-oxidant and chelating properties and their possible role in the prevention of chronic diseases (Schroeter et al., 2002).

Flavonoids are present in food mainly as glycosides and polymers (Hammerstone et al., 2000) and these comprise a substantial fraction of dietary flavonoids (Santos-Buelga and Scalbert, 2000). The biological properties of flavonoids are determined by the extent, nature, and position of the substituents and the number of hydroxyl groups (Schroeter et al., 2002). These factors also determine whether a flavonoid will act as an antioxidant or as a modulator of enzyme activity, or whether it possesses antimutagenic or cytotoxic properties. The most reported activity of flavonoids is their protection against oxidative stress (Rice-Evans 2001). Thus flavonoids can scavenge peroxyl radicals, and are effective inhibitors of lipid peroxidation, and can chelate redox active metals, and thus prevent catalytic breakdown of hydrogen peroxide. However, under certain conditions, flavonoids can also display pro-oxidant activity and this is thought to be directly proportional to the total number of hydroxyl groups (Cao et al., 1997), and they have also been reported to modulate cell signalling (Schroeter et al., 2002).

17. **Role of medicinal plants in the prevention and treatment of cancer**

It has been well recognized that allopathic drugs are not without side effects as they exhibit severe toxicity on normal tissues. Therefore, worldwide research is going on to explore the best effective preventive agents from different sources. Recent pharmacological researches revolve around the urgency to evolve suitable chemotherapeutic agents for the treatment of tumors (benign and malignant) without having toxic effects (Pandey and Madhuri, 2006).

India is the largest producer of medicinal plants and is so called as the "Botanical garden of the World". Medical information referred in the old Indian literatures includes several medicinal herbs, which have been in the use for thousands of years, in one form or the other, under the indigenous system of medicine. In India, 45,000 plant species have been identified, out of which about 15-20 thousand plants are of good medicinal value. However, traditional communities use only about 7000-7500 plants for medicinal purposes. In India, plant based medicines come under one system known as Ayurvedic system of medicine. In India, the Ayurvedic concept appeared and developed between 2500 and 500 BC. The literal meaning of Ayurveda is “science of life,” because ancient Indian system of health care focused views of man and his illness. According to Ayurveda, the disease evolves from the body due to external factors. Ayurvedic medicines mainly
based on plants enjoy a respective position today, especially in the developing countries, where modern health services are limited. In Ayurvedic system around 314 plants have been listed, which has been used in India. In the present context, the Ayurvedic system of medicine is widely accepted and practiced not only in the Indian Peninsula but also in the developed countries such as Europe, United States and Japan (Veale, et al., 1992). Plant derived medicines have been the first line of defense in maintaining health and combating diseases. In the last century, roughly 121 pharmaceutical products have been discovered based on the information obtained from the traditional healers (Anesini and Perez, 1993). Chemical principles from natural sources have become much simpler and have contributed significantly to the development of new drugs from medicinal plants. Biologically active compounds from natural sources have always been of great interest to scientists working on various dreadful diseases including cancer (Cox and Balick, 1994).

More than 50% of all modern drugs in clinical use are of natural products, many of which have been recognized to have the ability to include apoptosis in various tumour cells (Rosangkima and Prasad, 2004). According to the World Health Organization (WHO) estimates, more than 80% of the people in developing countries depend on traditional medicine for their primary health needs (Madhuri and Pandey, 2008; Sivalokanathan, 2005). Some medicinal plants and their products including vegetables, fruits and crops play an important role in cancer prevention. Consumption of large amounts of vegetables and fruits can prevent the development of cancer. Doctors recommend that people wishing to reduce their risk of cancer should eat several pieces of fruits and several portions of vegetables every day. Many plant-derived products exhibit potent antitumour activity against several cancer cell lines (Polidori, 2003).

17.1. **Cancer Chemoprevention**

The term chemoprevention was coined by Michael Sporn in 1976 to define the inhibition or reversal of carcinogenesis by the use of natural, synthetic or biological chemical agents that protect against the development and progression of mutant clones of malignant cells (Sporn, 1976). The concept of chemoprevention recognizes that in reality, cancer is not simply caused by a single threshold event; rather, it is an evolving multistep molecular and cellular process, which is characterized by a dormant period of many years between the initiation of carcinogenesis and the onset of the invasive and metastatic phases of the disease (Wattenberg, 1985; Sporn, 1991).

Cancer may arise as a result of chemical, physical, biologic, and/or genetic insults to cells. Specific external causative factors include smoking, occupational and environmental
chemicals, radiation, dietary factors, and specific viruses may initiate the process (Weinstein, 1991).

In addition, genetic factors have an important influence on an individual’s susceptibility to cancer development. Because the multistep nature of carcinogenesis includes an initiation step that involves changes at the genetic level followed by a number of promotion and progression steps that lead to malignancy, opportunities exist for intervention at early as well as later stages of the process, as indicated in the figure 21. (Wattenberg, 1978; Wattenberg, 1985; Weinstein, 1991; Garewal and Meyskens, 1991)

In general, chemopreventive agents may be classified by the point in the carcinogenic process at which they are effective: (1) compounds that prevent the formation of active carcinogens (initiation); (2) blocking agents that prevent carcinogens from reaching or reacting with cellular targets (initiation); and (3) suppressing agents that suppress the expression of neoplasia in cells exposed to doses and durations of carcinogens that otherwise would cause cancer (promotion). Some inhibitors have been found to have both blocking and suppressing capabilities (Wattenberg, 1985; Wattenberg, 1990).

**Figure 21:** Diagram of the stages of carcinogenesis and the opportunities that exist for intervention using chemopreventive agents.
Potential chemopreventive agents being investigated are diverse with respect to source, chemical structure, and physiologic effects and include micronutrients such as vitamins (e.g., folic acid and vitamins A, C, and E); minerals (e.g., selenium, molybdenum, and calcium); natural products (e.g., carotenoids, isothiocyanates, and flavonoids); and synthetics (e.g., vitamin A or D derivatives, piroxicam, tamoxifen, 2-difluoromethylornithine (DFMO), and oltipraz). The most widely studied class of chemopreventive agents is the retinoids-natural and synthetic derivatives of vitamin A (retinol) (Lippman et al., 1991).

Retinoids are potent regulators of cell differentiation and proliferation in essentially all epithelia that are sites for the development of invasive carcinoma. They are also able to restore some carcinogen-induced premalignant lesions to a more differentiated state. (Sporn, 1991; Sporn and Newton, 1979; Sporn and Roberts, 1983)
Figure 22: Proposed mechanism of certain chemopreventive agents (Tamimi et al., 2002)

“The field of chemoprevention has progressed to the point where chemoprevention is now considered to be an extremely promising approach to the prevention of invasive cancer”.
17.2. Mechanism of chemopreventive agents in cancer

Cellular and molecular events modulated or regulated by chemopreventive agents include modulation of phase I/II metabolizing enzymes, DNA repair, cell cycle progression, cell proliferation, differentiation and apoptosis, growth hormonal activity modulation, ligands for nuclear receptor, and modification of chromatin structure. Most of these mechanisms are interconnected or partially overlap with each other. Modulation of a given end point may be the result of a specific mechanism or the consequences of other upstream mechanisms (Flora and Ferguson, 2005). Many studies have been carried out on elucidating the molecular mechanisms of chemopreventive agents. Some of the mechanisms of action are described as under:

17.2.1. Modulation of phase I and II enzymes by chemopreventive agents

Metabolism of exogenous chemical substances in the body (xenobiotics) is generally divided into phase I and phase II metabolisms. Chemical carcinogen may be activated via phase I metabolism reactions (activation of pro-carcinogens to highly reactive carcinogens) or detoxified by phase II metabolism (involves conjugation process which increases polarity of the compounds thereby facilitating elimination). The physiological balance of these drug metabolizing enzymes between competing activating and detoxifying reactions determines the sensitivity of an individual towards carcinogens. Thus, modulation of phase I and phase II enzymes by phytochemicals can confer protection against carcinogen induced cellular damage (Issa et al., 2006).

Activation of antioxidant response element, a promoter region found in several genes encoding for detoxifying enzymes like NAD(P)H:quinone oxidoreductase-1 (NQO1), Heme oxygenase-1 (HO-1), Glutathione-S-transferase (GST) and Superoxide dismutase (SOD), has been used to screen for potential enzyme inducers. Transcription factor Nrf2, member of the basic leucine zipper NF-E2 family of transcription factors is known to bind and activate antioxidant response element. Nrf2 is bound in cytoplasm to Keap1, and following dissociation Nrf2 migrates into the nucleus and enhances gene transcription through binding to antioxidant response element. Destabilizing the Nrf2-Keap1 complex is a potential mechanism targeted by detoxifying enzymes inducers (chemopreventive agents) to activate cytoprotective enzymes expression (Yu and Kensler, 2005).
17.2.2. Antioxidant activity by chemopreventive agents

Reactive oxygen species generated oxidative stress may result in dysfunctional cell growth, differentiation, and death, which often occurs together with DNA mutations and ultimately leads to cancer development. Phytochemicals with antioxidant activities may exert their effects by absorbing free electrons and radicals. Compounds with hydroxyl groups attached to aromatic rings create an electron rich environment that traps the ROS, preventing them from reacting with nucleophilic centers of cellular proteins and DNA (Issa et al., 2006).

Antioxidants target free radicals, which maybe generated from normal oxygen metabolism or during inflammatory responses. ROS may contain odd numbers of electrons e.g. superoxide (O2\textsuperscript{-}), hydroxyl (OH\textsuperscript{-}), hydroperoxyl (HOO\textsuperscript{-}), peroxyl (ROO\textsuperscript{-}), and alkoxyl free radicals (RO\textsuperscript{-}), or even number of electrons such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and lipid hydroperoxide (ROOH). Redox sensitive transcription factors like NF-κB and AP-1 may be targeted by antioxidants since their activation promotes transcription of genes involved in cell cycle progression and cell proliferation (Loo, 2003).

17.2.3. Anti-inflammatory action of chemopreventive agents

Arachidonic acid metabolism is the link to inflammation. Archidonic acid is mainly catalyzed by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 into eicosanoids metabolites. The eicosanoids are lipid signaling mediators that play a central role in pathophysiological conditions. They have been identified as active carcinogens or tumour promoters (Hyde and Missailidis, 2009). Chemopreventive agents exhibiting anti-inflammatory properties may target archidonic acid dependent pathway or archidonic acid independent pathway. COX, LOX, and phospholipase A2 are considered archidonic acid dependent while nitric oxide synthase (NOS), 5-LOX, activating peroxisome proliferator activator receptor (PPAR), NSAID activated gene-1 (NAG-1) and NF-κB are classified as archidonic acid independent (Hyde and Missailidis, 2009).

The major mechanism of anti-inflammatory synthetic drugs or natural compounds relies primarily on their ability to inhibit the cyclooxygenase activity of the COX enzymes. COX-2 is the inducible form of COX which has a role in inflammatory and proliferative reactions (Kundu and Surh, 2008). Pro-inflammatory mediators such as growth factors (epidermal growth factor, transforming growth factor-β, and vascular endothelial growth factors), cytokines (TNF-α and IL-6), oncogenes (p53), and other factors that induce COX expression as well as the products of the COX and LOX pathways like
prostaglandins, thromboxanes, leukotrienes are also targeted by anti-inflammatory agents (Murakami and Ohigashi, 2007).

17.2.4. Modulation of cell signalling pathways by chemopreventive agents

Cellular signalling is a complex signal communication network in cells which controls basic biological activities and coordinates cell actions. The growth of cancer cells depends on multiple pathways. Altered proteins resulting from the mutations or defects of genes influence the way they communicate with each other. Chemopreventive agents like soy isoflavones including genistein and daidzein as well as indole-3-carbinol (I3C) and its dimeric product 3, 3-diindolylmethane (DIM) from cruciferous vegetables target the NF-κB, phosphoinositide 3-kinase (PI3K)/Akt, and MAPK pathways (Sarkar and Li, 2004).

Cellular targets such as NF-κB, IκB, and IKK control cell proliferation, apoptosis, inflammation, stress response within the NF-κB pathway; phosphoinositide-dependent kinase 1 (PDK1) and PDK2 activate the Akt pathway, which play critical roles in cell survival. MAPK pathways consist of a three-tiered kinase core in which a MAPK kinase kinase (MAP3K) activates a MAPK kinase (MAP2K) that activates a MAPK (ERK, JNK, p38), regulate cell growth and survival. Other molecular targets like Notch receptors, p53 protein as well as androgen receptors are involved in their respective pathways in cell regulation. All of these molecular players are targeted by chemopreventive agents in cancer chemoprevention (Dorai and Aggarwal, 2004).

17.2.5. Induction of apoptosis and cell cycle arrest

Insensitivity in induction of apoptosis and absence of normal cell cycle control mechanism promote uncontrolled cell proliferation. Many chemopreventive agents induce apoptosis through the mitochondria mediated pathway. Stress signals elicited by these chemopreventive compounds regulate pro-apoptotic proteins (e.g. Bax and Bak) or anti-apoptotic proteins (e.g. Bcl-2 and Bcl-x), leading to the release of cytochrome c from the mitochondrial inner membrane, followed by formation of ‘apoptosome’ (formed by cytochrome c, apoptotic protease-activating factor 1 (APAF-1) and caspase 9). Caspase 9 further activates downstream effector caspases, such as caspase-3, 6 and 7, which degrade important intracellular proteins, leading to the morphological changes showing the phenotype of apoptotic cells. Meanwhile, disturbance of the balance among cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs), governing the progression of the
cell cycle by chemopreventive compounds can potentially inhibit proliferation of neoplastic cells (Chen and Kong, 2005).

Some chemopreventive agents activate upstream kinases such as JNK or inhibit PI3K/Akt pathway to induce apoptosis, inhibiting NF-κB and AP-1 activation, thereby down-regulating anti-apoptotic and cell cycle regulating proteins, inducing caspase activation to execute cell death and increasing p53 expression eliciting cell cycle arrest through the induction of CDKIs (p21 and p27) and the inhibition of CDK4, CDK2, cyclin D1 and cyclin E (Chen and Kong, 2005).

17.3. Examples of some chemopreventive agents

17.3.1. *Glycyrrhiza glabra* L. (Yashti-madhu, Licorice)

Licorice is a versatile medicine in India and China, for gastrointestinal health. It is a mild laxative, which soothes and tones the mucous membranes and relieves muscle spasms. Clinical studies have proved that the Licorice extracts to be more effective as well known synthetic alternatives. It is rich in flavonoids and pentacyclic triterpene saponin as major constituents, which include liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin and glycyrrhizic acid (Kim *et al*., 2004). It possesses numerous biological activities including antioxidant, Hepatoprotective, anti-inflammatory, anti-cancer activities. Licorice has held claim for therapeutic use for fevers, liver ailments, dyspepsia, gastric ulcers, sore throats, asthma, bronchitis, Addison’s disease and rheumatoid arthritis and has been used as a laxative, antitussive and expectorant (Wang *et al*., 2000). Its mode of action is as an anti-mutagen, preventing damage to genetic material that can eventually result in its anti-cancer property (Rahman and Sultana, 2006; Cheel *et al*., 2010).

17.3.2. *Terminalia chebula* Retz. (Haritaki, Chebulic myrobalam)

*Terminalia chebula* is an important medicinal plant in Indian traditional medicine and it is most frequently used herb in Ayurveda. *Terminalia chebula* is called the “King of Medicine” in Tibet and is always listed at the top of the list in Ayurvedic Material due to its extraordinary power of healing. The dried ripe fruits of Terminalia chebula have traditionally been used in the treatment of asthma, sore throat, vomiting, hiccup, diarrhoea, bleeding piles, gout, heart and bladder diseases (Kirtikar and Basu, 1935). Terminalia chebula is routinely used as traditional medicine by tribals of Tamil Nadu in India to cure several ailments such as fever, cough, diarrhoea, gastroenteritis, skin diseases, candidiasis, urinary tract infection and wound infections (Dash, 1991). Recently it has been demonstrated that *Terminalia chebula* exhibit anti-tumor promoting efficacy (Prasad *et al*.,
2006). Anti-cancer property of Terminalia chebula is thought to be through the inhibition of ornithine decarboxylase (ODC) and down regulation of NF-κB (Das et al., 2011).

17.3.3. *Withania somnifera* (Linn.) Dunal (Ashwagandha)

*Withania somnifera* commonly known as Ashwagandha is one of the best Ayurvedic herbs and holds a place in the Ayurvedic traditions similar to Ginseng in Chinese therapies. It has been often referred to as the "Indian Ginseng". Western research supports its polypharmaceutical use, confirming antioxidant, anti-inflammatory, immune-modulating, and anti-stress properties in the whole plant extract and several separate constituents (Mishra et al., 2000). As an antioxidant, *Withania somnifera* and its active constituents sitoindosides VII-X and withaferin A have been proven to increase levels of endogenous superoxide dismutase, catalase, and ascorbic acid, while decreasing lipid peroxidation (Bhatnagar et al., 2005). *Withania somnifera* acts as an anti-inflammatory agent through inhibition of complement, lymphocyte proliferation, and delayed-type hypersensitivity. *Withania somnifera* modulates the immune response, increasing the expression of T-helper 1 (Th1) cytokines, as well as CD4 and CD8 counts, and natural killer (NK) cell activity (Bani et al., 2006; Khan et al., 2006). It also possesses anti-cancer properties (Prakash et al., 2001).

17.3.4. *Zingiber officinale* Rosc (Sunthi, Ginger)

Ginger (Zingiber oficinale Roscoe, Zingiberaceae) is widely used as a dietary species throughout the world. Besides its extensive utilization as a spice, the rhizome of ginger has been used traditional medicine to ameliorate such symptoms as inflammation, rheumatic disorders and gastrointestinal discomforts. Ginger is used extensively in traditional Chinese medicine to treat headaches, nausea and colds and in Ayurvedic and western herbal medicinal practice for the treatment of arthritis, rheumatoid disorders and muscular discomforts. Ginger is often used for the treatment of stomachache, and cardiovascular and motor diseases (Dedov et al., 2002). It also possesses anti-inflammatory activity and regulates bacterial growth, as well as providing protection for immune-depressed patients (Penna et al., 2003). This species contains biologically active constituents including the main pungent active principles, the gingerols and shogaols (Korikanthiman et al., 2002).

17.3.5. *Butea monosperma*

*Butea monosperma* (Palash), traditionally employed intensively as folklore remedy for a wide spectrum of liver diseases in India. It has a wider array of uses than any other herb. Numerous scientific reports validate the traditional uses of *B. monosperma* in the
maintenance of general health. Practically every part of *B. monosperma* (leaves, bark, fruit, flowers, oil, and gum) have been reported to be associated with various remedial properties such as, anti-diarrhoeal (Gunakkunru *et al*., 2005) and antiestrogenic effects (Shah *et al*., 1990), acceleration of cutaneous wound healing (Sumitra *et al*., 2005). Recently our laboratory has reported the chemopreventive potential of *B. monosperma* on thioacetamide induced early tumor promotion related events in rat liver (Sehrawat *et al*., 2006). The important active principles of *B. monosperma* are butin, butein, butrin, isobutrin, palasitrin, coreopsin and isocoreopsin, chalcones, and aurones triterpene phenolics constituent (Gupta *et al*., 1970).

Indian systems of medicines may be explored with the modern and most sophisticated scientific approaches for better leads in the health care. The development of these traditional systems of medicines with the perspectives of safety, efficacy and quality will help not only to preserve this traditional heritage but also to rationalize the use of natural products in the health care. **In the light of the literature available and present status of the liver cancer chemoprevention, the main objectives of the present study were as follows:**