REVIEW OF LITERATURE
Cancer today tops the list of the killer diseases affecting humankind. Continuous research for decades has still not provided us a complete understanding of this disease and hence a cure. Cancer describes a subset of lesions of the disease neoplasia. A ‘neoplasm’ is defined as a heritably altered relatively autonomous growth of tissue. Neoplasm’s may be either benign or malignant. The distinction between these classes is related to the characteristic of successful metastatic growth of malignant, but not benign neoplasms. Metastases are secondary growths of cells from the primary neoplasm. Cancers are malignant neoplasms whereas the term ‘tumor’ describes space-occupying lesion that may or may not be neoplastic. The process of generation of cancer is known as ‘carcinogenesis’ and a “carcinogen” is an agent whose administration to previously untreated animals leads to a statistically significant increased incidence of neoplasms of one or more histogenetic types as compared with the incidence in appropriate untreated animals (Pitot 1986).

2.1 Causes for cancer:

There are various endogenous as well as exogenous causes for cancer. The endogenous causes include heridity, hormonal imbalances, and free radicals generated during metabolism that are capable of damaging the DNA. The exogenous causes include exposure to known carcinogens including cigarette smoke, viruses, and occupational or environmental exposure to certain carcinogens and faulty dietary habits. The exogenous causes can be well taken care since these are external stimuli.

2.1.1 The endogenous causes

Some cancers have a genetic origin and run in a family. Certain breast, colon cancers, melanomas, retinoblastoma, kidney cancers and neuroendocrine cancers are a result of inherited mutations of specific genes. Endogenous as well as exogenous hormones drive cell proliferation, and thus provide an opportunity for the accumulation of random genetic errors. Most
cancers of the breast, endometrium, ovary, prostate, testis, thyroid and osteosarcoma are hormone related. Estrogens are known to induce and promote mammary tumors in rats and exposure to estrogens unopposed to progestins increases the risk of endometrial tumors (Henderson and Feigelson, 2000). Animal studies have repeatedly demonstrated that estrogens can induce and promote mammary tumors in rodents and also that progestins are breast mitogens and as such are likely to increase breast cancer (Dao, 1981; Cline et al. 1996). Several gynecological factors are responsible for cancer generation. Early menstruation is a major risk factor for breast cancer and so is late age at first pregnancy. Early age at menarche and late age at menopause, also increase the chances of breast cancer. More the number of offspring’s the woman has, the less likely she is to suffer from the cancer of the endometrium, breast or ovary.

Some chemicals interfere with the hormonal pathways of the endogenous hormones. The evidence for the female hormones as carcinogenic agents is stronger than the male hormones and there is also evidence for synthetic androgens as carcinogens. Hormones can also act along with other agents to induce neoplasia. Ip et al. (1980) demonstrated that high levels of endogenous prolactin enhance the induction of mammary tumors in rats by dimethylbenz(a)anthracene. Cellular metabolites including oxygen free radicals, nitric oxide, peroxynitrite and the hydroxyl can react with the DNA to form oxidized bases e.g. 8-oxodeoxyguanosine. Malondialdehyde, a major aldehyde product of lipid peroxidation is mutagenic in bacterial and mammalian cells and carcinogenic in rats.

Angiogenesis is important for the growth of solid tumors and starving the cancer cells is a safer therapeutic approach to control the growth of a tumor (Kerbel, 2000). Physiological traits to a certain extent make a person more susceptible to cancers, For example fair skin makes a person more prone to skin cancers. Also if a person inherits a normal genetic variant that causes the body to eliminate certain carcinogens relatively inefficiently, that person after
repeated exposure to the carcinogen will be more likely to develop the cancer, than a person who has an efficient form of the gene. The BRCA1, BRCA2, p53 genes are associated with breast cancers. MSH2, MSH1, PMS1, 2 and APC genes are associated with colon cancers. MTS1, (CDKN2) and CDK4 are associated with melanomas. The RB gene is well known and is associated with retinoblastoma and the WT1 and VHL are associated with kidney cancers. Regarding the neuroendocrine cancers the NF-1, NF-2 and RET genes are known to be involved (Trichopoulos et al. 1996). Inherited mutations are rare and less than 5% are fatal cancer cases, since evolutionary pressure assures that such mutations stay at a minimum.

2.1.2 The exogenous causes

The exogenous causes include agents that may directly react with the cellular DNA causing permanent damage to it. These are known as initiating agents. Some chemicals alter only the expression of the cellular DNA and do not react with it. These are the promoting agents. Another group of chemicals can cause the initiated or the cells in the promotion stage to progress to a cancer and these are known as progressors.

There exists a variety of causes for cancer. Apart form a person being exposed to external agents, the life-style of the person is one of the important governing factors in determining the risk an individual possesses in contacting a cancer. Smoking, alcohol, lack of exercise, excessive caloric intake and a sedentary life style all make a person prone to a risk for cancer. Cigarette smoke contains a variety of nitrosamines and tar components known to induce carcinogenesis (Hecht, 1985) whereas alcohol and excessive calories can promote initiated cancers. People consuming animal (saturated) fat and red meat are more susceptible to the cancers of the colon and rectum. The life style related causes do not act directly, but only enhance the cancer risk by affecting other pathways such as the hormonal or DNA damage due to excessive generation of free radicals. Exposures to carcinogenic chemicals at the work place are well known causes of cancer. Exhaust of vehicles contains a mixture
of chemicals that are known environmental pollutants with a carcinogenic potency.

A wide variety of chemicals are known to cause cancers. It was first described by Yamagawa and Ichikawa in 1915 that the application of coal tar to the skin of animals produces skin tumors (Pitot and Dragan, 2001). Later the active principles such as benzo(a)pyrene (3,4- benzpyrene) in coal tar were isolated and several other polycyclic hydrocarbons were synthesized with known carcinogenic potency. Several chemicals act at sites other than the site of contact. The aromatic amine 2-naphthylamine and several other aromatic amines are carcinogenic for the urinary bladder in humans (Vainio et al, 1991). The dialkyl nitrosamines are potent carcinogens of the liver and kidneys (Schmahl and Habs, 1980). Certain metals such as beryllium, cadmium, cobalt, chromium, nickel, iron, arsenic, lead, titanium and zinc have been shown to be carcinogenic in experimental animals (Verschaeve et al. 1979; Kuschner, 1981; Sky-Peck, 1986). There exists a large list of inorganic, organic chemicals and metallic elements, which are known to cause cancer.

Certain chemicals do not react with the DNA but are effective in promoting a cancer. Such chemicals known as epigenetic carcinogens form a major group. They provide favorable conditions for the growth of genetically altered cells. A major characteristic of this stage is its reversible nature. Discontinuation of the application of the promoting agent leads to the regression of the tumor. The exact mechanisms by which these agents act are yet to be elucidated.

2.1.2.1 Genetic alterations due to exogenous chemicals

Two sets of genes play major roles in triggering the growth of cancer. These are the proto-oncogenes class that encourage the growth of a tumor and the tumor suppressor genes that inhibit the growth of a tumor. When mutated, the proto-oncogenes can become carcinogenic oncogenes that drive the cells towards excessive multiplication. The best understood example of mutated genes and faulty signaling comes from the \textit{ras} family of oncogenes. The proteins encoded by the normal \textit{ras} genes transmit stimulatory signals from
growth factor receptors to other proteins further down the line. The proteins encoded by the mutant ras genes however fire continuously. The myc family of oncogenes alters the activity of transcription factors in the nucleus. The myc transcription factors are normally synthesized only when the cell surface growth receptors are stimulated. The myc proteins activate genes that force the cell's growth forward.

The TGF-β can stop the growth of various types of normal cells. Some colon cancer cells become oblivious to TGF-β by inactivating the gene that encodes a cell surface receptor for this substance. The tumor suppressor genes contribute to cancer when they are inactivated by mutations. Bcl-2, an anti apoptotic gene, promotes cell survival, Over expression of this gene has shown to promote lymphoproliferation and accelerated c-Myc induced lymphomagnesis. Bcl-xl, a potent death suppressor is up regulated in some tumor types. The death promoter Bax is inactivated in certain colon cancers and in hematopoietic malignancies (Bishop, 1995). p53 was the first tumor suppressor gene linked to apoptosis and could induce apoptosis when over-expressed in myeloid leukemia cell line. Little is known of the involvement of caspase mutations in cancer. Mutations in p53 result in decreasing the stringency of DNA damage checkpoint, allowing cells to replicate the damaged DNA and accumulate mutations in daughter cells that lead to genomic instability (Weinberg, 1996).

2.2 The multi-step nature of carcinogenesis

The DNA of a cell is a chemically reactive substance and combines with substances that can react with it. A large number of chemicals or their reactive metabolites react with the DNA resulting in DNA-adduct formation. Cells have the ability to repair the damaged DNA. The bulky adducts can be easily repaired since they are detected easily but smaller adducts may not be detected and hence not repaired. This mis-incorporation of the nucleotides may lead to mutations. Under the influence of DNA polymerase, the cell divides carrying the mismatch pair to the next generation. A single mutation may not be sufficient for the cell to gain autonomy. The nucleotide sequence of cellular
DNA is maintained at a homeostatic equilibrium, such that an increase in the production of DNA damage or reduction in DNA repair results in an increased frequency of mutations (Loeb and Loeb, 2000). After 2-3 generations, several mutations may occur and the cell may gain complete autonomy, leading to a cancer. Thus the process of generation of cancer is a multistage process proceeding through hyperplasia to cancer.

The development of cancer progresses through successive stages involving tumor initiation, promotion and progression. Berunblem and Shaubik in 1941 were among the first to demonstrated that skin carcinogenesis can be clearly divided into a stage of initiation produced by the direct action of the carcinogen and a more longer stage of promotion brought about by the repeated application of the promoting agent (Pitot and Sirica, 1980). An initiating agent can be defined as a physical, chemical or a biological agent that is capable of directly altering in an irreversible manner the native molecular structure of the genetic component (DNA) of the cell. One of the most important characteristics of initiation is that a cell, once initiated, does not lose this induced property with time. A promoting agent can be defined as an agent that enhances the growth of an initiated cell without further altering the genetic component of the cell. Promoting agents may alter the expression of genetic information of a cell. These agents do not directly act on the genetic material but rather affect its expression by a variety of mechanisms including their interaction with the cell surface receptors or with cytoplasmic and nuclear protein receptors, or by an alteration of other cellular components and functions (Pitot and Sirica, 1980).

2.2.1 Initiation:

The molecular mechanisms of initiation must conform to the observable characteristics of this stage. At least three processes are important in initiation: metabolism, DNA reactivity of metabolite, DNA repair and cell proliferation. Initiated cells are difficult to distinguish morphologically and phenotypically from their normal counterparts and the molecular mechanisms too are difficult to identify. Sub-carcinogenic doses of initiating agents may induce substantial
DNA alkylation and are capable of initiating cells in experimental models of multistage carcinogenesis (Pegg and Perry, 1981; Bichara and Fuchs, 1985). The ras gene codes for guanosine triphosphatases, which function as a molecular switch for signal transduction pathways involved in the control of growth, differentiation and other cellular functions. Several classes of genes appear to be appropriate targets for DNA damaging carcinogens. The actual role of proto-oncogene and cellular oncogene mutations in establishing carcinogenesis is not clear. Initiating agents’ form adducts with DNA. Alkylating agents causes the addition of an alkyl group to DNA. The initial step in the conversion of aromatic amines to tumor forming metabolites involves N-hydroxylation. The alkylating agent N-nitrosodiethylamine (DEN) is metabolized in two steps to first yield a hydroxylated form of DEN which later splits to produce an electrophilic ethyl ion. The directly acting alkylating agents induce preferential binding to highly nucleophilic centers such as the N7 position of guanine. The ethyl ion can also react with the nucleophilic oxygens in DNA. The position of an adduct in the DNA and its chemical and physical properties in that context dictate the types of mutations induced (Essigman and Wood, 1993).

The CYP2A6 isoform of cytochrome P450 is responsible for the metabolism of DEN to its ultimate carcinogenic form, the ethyl ion. The ethylation of the DNA by DEN causes a mutation of the C A/61 or A C in the Ha-ras gene in the mouse liver (Bauer-Hoffman et al. 1992). The presence of certain alkylation products, such as O-alkyl deoxyguanosine and O-alkyl deoxythymidime, permits a degenerate base pairing that can base pair with an appropriate base as well as with an inappropriate base. Thus alkylating agents result in mutations as a result of base mispairing.

Ethylation of DNA occurs predominantly in the phosphate backbone. Swenberg et al (1984) reported that O-alkyl thymine may be more important adduct for carcinogenesis because this DNA adduct is retained in the DNA for more extended periods than is O-alkyl guanine adduct. Also the O-alkyl thymine is stable in liver parenchymal cells than the O-alkyl guanine adduct
after continuous exposure of the rats to DEN. The hypo-methylated genes are actively transcribed, whereas the hyper-methylated ones rarely tend to be transcribed.

Because a mutation may arise during the synthesis of a new DNA strand through the use of the damaged template, cell replication becomes an important factor in the fixation of a mutation. A high rate of cell division tends to enhance both the spontaneous and the induced level of mutation through the chance inability of the cell to repair DNA damage before DNA synthesis (Umar and Kunkel, 1996)

2-Acetylaminofluorene (AAF) is a known carcinogen and it has been proved that the metabolite of this agent is responsible for its carcinogenic effect. Miller et al. (1960) reported the formation of N-hydroxy metabolite of AAF in the rats and that this metabolite is more carcinogenic than the parent compound. In contrast to the N-hydroxylation, the ring hydroxylation of 2-AAF leads to a complete loss of carcinogenic activity. The N-hydroxylation of AAF can be followed by esterification of the N-hydroxyl group to yield a highly reactive compound that is capable of non-enzymatic reaction with nucleophilic sites on proteins and nucleic acids. The ultimate carcinogenic form of AAF also reacts with guanine at two positions on the DNA base. The AAF-DNA adduct distorts the double helix and induces frameshift mutations (Bichara and Fuchs, 1985). It was also demonstrated that whereas 2-AAF is not mutagenic, its sulfate metabolite is highly mutagenic for transforming DNA (Maher et al. 1968). The 3-((deoxyguanosine)-N²-yl)-acetylaminofluorene adduct is extremely difficult for the cell to repair and hence AAF can produce a wide spectrum of neoplasms. The CYP1A2 isoform of the CYP450 oxidases is responsible for the metabolism of AAF.
Table 1: Molecular and cellular mechanisms in multi-stage carcinogenesis

<table>
<thead>
<tr>
<th>Initiation</th>
<th>Promotion</th>
<th>Progression</th>
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<tbody>
<tr>
<td>Simple mutations (transitions, transversions, small deletions, etc.)</td>
<td>Reversible enhancement or repression of gene expression mediated via receptors specific for the individual promoting agent.</td>
<td>Complex alterations (chromosomal translocations, deletions, gene amplification, recombination, etc.) resulting from evolving karyotypic instability.</td>
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<tr>
<td>involving the cellular genome.</td>
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</tr>
<tr>
<td>In some species and tissues, point mutations in protooncogenes and/or potential cellular oncogenes.</td>
<td>Inhibition of apoptosis by promoting agent.</td>
<td>Irreversible change in the gene expression, including fetal gene expression, altered major histocompatibility complex (MHC) gene expression, and ectopic hormone production.</td>
</tr>
<tr>
<td>Mutation in genes of signal transduction pathways that may result in an altered phenotype.</td>
<td>No direct structural alteration in DNA from action or metabolism of promoting agent.</td>
<td>Selection of neoplastic cells for optimal growth genotype/phenotype in response to the cellular environment and including the evolution of karyotypic instability.</td>
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2.2.2 Promotion:

Several agents are known to promote the growth of the initiated cells. Receptors for some of the agents are known but the in most cases even today the mechanism for the promotion is not known. A variety of chemicals have been shown to induce promotion and there is no evidence that promoting agents or their metabolites directly interact with DNA or their metabolism is required for their effectiveness. The distinctive characteristic of promotion is the reversible nature of this stage. Another characteristic of promotion is promoting agents exhibit a dose response relationship with a sigmoid curve (Ashendel, 1985), though there may be exceptions.

Boutwell (1974) first proposed the theory that promoting agents may induce their effects through their ability to alter gene expression. The regulation of genetic information is mediated through recognition of xenobiotics and their specific molecular interaction with either a surface or cytosolic receptor. For example, estrogen and androgen receptors for the sex
steroids and the peroxisome proliferator activated receptor (PPAR) for the peroxisome proliferators are involved in these aberrant responses. Agents such as 2-AAF exert mito-inhibitory effects and is an effective tumor promoter in the initiation-selection protocol, where it inhibits the growth of the normal liver parenchyma, but the altered cells being resistant to the mito-inhibitory effect of 2-AAF metabolite(s) continue to proliferate.

Tetradecanoyl phorbol acetate (TPA) is a naturally occurring alicyclic chemical present in croton oil, a promoting agent used for the promotion of mouse skin tumors. Saccharin is an effective promoting agent for the bladder and phenobarbital is an effective promoter for hepatocarcinogenesis.

Phenobarbital, a well-known promoter of hepatocarcinogenesis, predominantly induces preneoplastic lesions that are eosinophilic in appearance. In addition, it significantly increases DNA synthesis in focal hepatocytes and decreases the rates of apoptosis in focal hepatocytes. (Kalaja et al. 1996). Phenobarbital is also shown to inhibit cell to cell communication (Whysner et al. 1996). Scholz et al. (1990) observed increases in the reactive oxygen species after chronic phenobarbital treatment to rats initiated with DEN, which may be related to its promotion potential. Arora et al. (1989) reported the synergistic effect of diethylstilbestrol and phenobarbitone in the production of pre-neoplastic lesions in rats. Connexin32 (Cx32) is the major gap junction forming protein in liver and functional Cx32 is required for the promoting effects of phenobarbital (Moennikes et al. 2000). Many tumor-promoting agents, including phenobarbital, block gap junction intercellular communication (GJIC) in vitro, and it has been suggested that this effect is relevant for clonal expansion of neoplastic cells in vivo. Phenobarbital has also been shown to inhibit apoptosis in c-myc hepatocytes but not in wild-type hepatocytes; it also decreased p53 and bax, and increased bcl-2 protein levels (Christensen et al. 1999). Tetrachlorodibenzo-p-dioxin (TCDD) is probably the most effective promoter for liver but is also effective in the lung and skin.

Estradiol is a representative of endogenous hormone that is an effective promoting agent. Both natural and synthetic androgens and estrogens are
effective promoting agents in their target end organs as well as in the liver (Sumi et al. 1980; Kemp et al. 1989). Cholic acid enhances preneoplastic and neoplastic lesions in the rat colon (Magnuson et al. 1993) whereas 2,2,4-trimethylpentane and unleaded gasoline effectively promote renal tubular cell tumors in rats (Short et al. 1989). Wy-14643, nafenopin and clofibrate and other lipid lowering drugs belong to a class of compounds known as peroxisome proliferators that induce the production of peroxisomes in the liver. These are effective promoting agents and induce hepatic neoplasms on long term administration at high doses to rodents (Reddy and Lalwani, 1983). Several other agents belonging to a diverse class such as polypeptide hormones, dietary factors including total calories, halogenated hydrocarbons have been also found to enhance the development of preneoplastic and neoplastic lesions in one or more systems of carcinogenesis.

Boutwell (1964) first demonstrated that when the frequency of application of the promoting agent was decreased after initiation in mouse skin there was a lower yield of papillomas in comparison with that obtained by a more frequent application. The regression of the preneoplastic lesions after withdrawal of the promoting agents may be due to apoptosis and this proposed mechanism is supported by the demonstration that many promoting agents inhibit apoptosis (Schultz-Hermann et al. 1990, Schultz-Hermann et al. 1993). Another potential pathway of this operational reversibility is “redifferentiation” or remodeling (Tatematsu et al. 1983). Thus cells in the stage of promotion are dependent on the continued administration of the promoting agent (Hanigan and Pitot, 1985) as was implied by the early studies of Furth (1959) on hormonally dependent neoplasia.

Another characteristic of promotion is its susceptibility to modulation by physiologic factors. Promotion may be modulated by aging (Van Duuren et al. 1975), dietary or hormonal factors (Sivak, 1979). Phenobarbital acts to decrease the serum T₃ levels by stimulating enzymes that metabolize it, this mechanism is similar to effects of goiterogens which prevent T₃ formation and release from the thyroid (McClain, 1989). Similarly unleaded gasoline acts like...
an anti-estrogen, thus removing the estrogen protection usually provided against the development of liver neoplasms in mice (Standevan et al. 1994).

On the other hand, many modulating factors may themselves be promoting agents. Several hormones may be efficient promoters and thus may serve as an endogenous or exogenous source for the modulation of cell proliferation (Pitot, 1991). Promoters exhibit a dose response relationship with sigmoid like curves and an observable threshold and a maximal effect. The threshold effect of the promoting agents may be considered a consequence of the reversible nature of their effects at the cellular level. The maximal effect is due to saturation of ligand binding or promotion of all the initiated cells. The dosing frequency determines the end result of the incidence of preneoplastic lesions, since altering the schedule results in a very less incidence of the lesions. This reinforces the fact that the stage of promotion is operationally reversible and indicates that a threshold dose effect level may exist.

Receptor interactions in promotion

Boutwell (1974) first proposed the theory that promoting agents may induce their effects through their ability to alter gene expression. Receptors are present on the cell surface and the cytosol. Ligands (environmental effectors, hormones, and drugs) interact with the receptors and regulate the genetic expression. The receptor mechanisms may generally be classified into three broad classes, steroid, tyrosine kinase and G-protein linked. Many promoting agents exert their effects on gene expression through perturbation of one of these signal transduction pathways. The ligand-receptor interaction leads to a cascade of events mediated through a number of second messengers finally effecting the gene expression. TPA a well-known promoter of mouse skin tumors alters the genetic expression by acting through the G protein or tyrosine kinase receptors linked protein kinase C second messenger pathway (DiGiovanni, 1992). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) acts in the steroid pathway via a specific Ah receptor. Sex steroids, some synthetic antioxidants and peroxisome proliferators interact with specific soluble receptors and alter the receptor expression by presumed mechanisms similar to
that of TCDD. In some cases such as phenobarbital the receptors are not yet defined.

2-Acetylaminofluorene acts as a selection pressure-inducing agent for the enhancement of proliferation of altered hepatic neoplasms in an initiation-promotion protocol. 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 metabolite and continue to proliferate (Roomi et al. 1985). This hypothesis provides a partial explanation of the tissue specificity demonstrated by many promoting agents.

The principle mechanisms for promotion are

1. Differential inhibition: 2AAF creates a differential for the rapid growth of initiated (resistant) hepatocytes when a stimulus for hepatocyte proliferation is applied.

2. Differential stimulation: Here a promoter selectively stimulates that cell to grow. Some agents are general proliferators and induce hypertrophy and hyperplasia, but the uninitiated cells soon lose the ability to proliferate and a differential stimulus is generated.

3. Differential recovery: Some of the initiated cells may undergo reversion to the normal cells, whereas some of the cells may remain.

Several factors can alter the stage of promotion. Diet and chemicals, may enhance or regress the tumor promotion by an agent. In one study vitamin A deficiency enhanced the hepatocarcinogenesis in female SD rats initiated by DEN after partial hepatectomy and promoted by TCDD (Flodstrom et al. 1991).
Table 2: Morphological and biological characteristics of the stage of initiation, promotion and progression of carcinogenesis

<table>
<thead>
<tr>
<th>Initiation</th>
<th>Promotion</th>
<th>Progression</th>
</tr>
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<tbody>
<tr>
<td>Irreversible, Initiated stem cell not morphologically identifiable.</td>
<td>Optionally reversible both at the level of gene expression and at the cellular level.</td>
<td>Irreversible, Morphologically discernible alteration in cellular genomic structure resulting from karyotypic instability.</td>
</tr>
<tr>
<td>Efficiency sensitive to xenobiotic and other chemical factors.</td>
<td>Promoted cell population existence dependent on continued administration of promoting agent.</td>
<td></td>
</tr>
<tr>
<td>Spontaneous (endogenous) occurrence of initiated cells.</td>
<td>Efficiency sensitive to aging and dietary and hormonal factors. Endogenous promoting agents may effect spontaneous promotion.</td>
<td>Growth of altered cells sensitive to environmental factors during early phase of this stage.</td>
</tr>
<tr>
<td>Requirement for cell division for fixation.</td>
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<td></td>
</tr>
<tr>
<td>Dose response not exhibiting a readily measurable threshold.</td>
<td>Dose response exhibits measurable threshold and maximal effect.</td>
<td>Benign or malignant neoplasms observed in this stage.</td>
</tr>
<tr>
<td>Relative potency of initiators dependent on quantitation of pre-neoplastic lesions after defined period of promotion.</td>
<td>Relative potency of promoters measured by their effectiveness in causing an expansion of the initiated cell population.</td>
<td>“Progressor” agents advance promoted cells into this stage.</td>
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2.2.3 Progression

The third stage in the generation of cancer after initiation and promotion is ‘progression’. Progression results from the continuing evolution of a basically unstable karyotype. Malignant karyotype posses the following characteristics of growth rate, invasiveness, metastatic frequency, hormonal responsiveness, and morphological characteristics ascribed to as karyotypic instability (Pitot, 1993). Agents that act only to effect the transition of a cell from the promoting stage to the progression stage may be properly termed as progressor agents. These agents may have the property of inducing chromosomal aberrations but may not be capable of initiation. Some examples of progressor agents are: benzene, asbestos fibers, arsenic salts, benzoyl
peroxide, hydroxy urea and 2,5,2',5'-tetrachlorobiphenyl. Preneoplastic lesions are identified during the stage of promotion, whereas neoplastic lesions are identified in the stage of progression.

The stage of progression usually develops from the stage of promotion but may also develop directly from the initiated cells. This happens as a result of administration of relatively high, usually toxic doses of complete carcinogens capable of inducing both initiation and progression. Harris (1991) reported that the genetic instability of this stage is primarily a reflection of the karyotypic changes seen rather than point mutations or gene amplification. Disruption of the mitotic apparatus, alteration in telomere function, DNA hypomethylation, recombination, gene amplification, gene transposition and involvement of the mismatch repair genes, may lead to karyotypic instability.

2.3 Experimental Hepatocarcinogenesis:

The concept of stepwise unfolding of the carcinogenic process is primarily based on morphological observations and correlated cell behavior. Hepatocarcinogenesis in the rat has received particular attention for this purpose. The obvious advantage of the experimental model is the end effect closely resembles the human tumor. Secondly the stages of the process can be separately induced and studied once a phenotypic marker for their identification is available, and that such stages can be quantified. Thirdly, liver was found to be the major target organ (~67%) for 65 carcinogens has been evaluated by IARC and 204 compounds evaluated by National Toxicology Program (NTP) in rats and mice, the organ specificity of the rest of the compounds is: 52% are carcinogens for the liver, 23% for forestomach, 22% for mammary gland and lung, 14% for nasal cavity and 12% for urinary bladder (IARC monographs; 1987; Zeiger, 1987). This liver specificity in rodents was irrespective of the actual target organ affected in humans. Thus more than half of the chemical carcinogens rated as positive compounds are hepatocarcinogens. Liver is also the obvious choice as the best organ especially for evaluating pharmaceuticals since metabolism of drugs in liver plays a
crucial role not only in the pharmacological effects but also in manifestation of toxicity and carcinogenicity.

Cells in the stage of promotion are termed as preneoplastic since their existence is entirely dependent on the continued presence of the promoting agent in the environment. Histological alterations in the growth pattern preceding invasive neoplasia have long been recognized by pathologists and have been classed as benign or imperfect neoplasia or as pre-cancerous pre-malignant or pre-neoplastic lesions (Carter, 1984). Other well-known examples of such lesions are papillomas of the skin, hyperplastic nodules of the breast or liver, adenomas of the colon and carcinoma in-situ of the cervix uteri.

Historically, the two-year-long term in-vivo testing using rats, mice or hamsters has been considered to be the most reliable method for prediction of carcinogenic potential of chemicals in humans (Guidelines for carcinogen bioassay in small rodents, 1976; Long term and short term screening assays for carcinogens, 1980). However to be internationally accepted, long-term tests must satisfy costly regulatory guidelines for appropriate facilities, long duration (2 years), maintenance of animals, sufficiently large numbers of rodents and careful histopathological examinations. The vast number of compounds that have been introduced into our environment in the recent years is, thus far beyond our capacity to assess using such comprehensive carcinogenicity in-vivo tests in each case. Another disadvantage of the chronic bioassay is its inability to differentiate initiators from promoters. Models of short and medium term hepatocarcinogenicity have been introduced recently to combat this problem.

The major advantage of experimental hepatocarcinogenesis as a model system is the large amount of background information available on the morphogenesis of hepatocellular carcinoma (Williams, 1980) and the even larger body of data available on the biochemistry and physiology of the liver. The heterogeneity and instability of foci phenotype of pre-neoplastic lesions in the same target tissue has been studied most closely in animal models of hepatocarcinogenesis. Pre-neoplasia has been defined as a phenotypically
altered cell population which has no obvious neoplastic nature but which indicates an increased risk for the development of both benign and malignant neoplasia (Bannasch, 1986).

2.3.1 Models used for short-term hepatocarcinogenicity experiments

Scherer and Emmelot (1975) first described the effect of administering low doses of diethylnitrosamine (DEN) shortly before or after a partial hepatectomy. Pitot et al. (1978) reported 5- folds increase in the carcinomas in rats when DEN at 5 or 10mg/kg was administered within 24 hrs of partial hepatectomy subsequently followed by 0.05% phenobarbital in drinking water for next 6 months. Moreover when DEN alone was used without partial hepatectomy at a dose of 20mg/kg, no neoplasms developed. This indicates a clear distinction in between the initiation and promotion stages. These islands of altered hepatocytes or foci of resistant hepatocytes can be used as indices of initiation. It is thus evident that this very early or first step in carcinogenesis is at least a two step process – ‘induction’ of a biochemical event and ‘fixation’ of such change by a round of cell proliferation.

A controversy still exists over the cellular origin of hepatic neoplasia. Hepatocytes, cholangiolar (oval) cells and liver stem cells have been proposed to be the possible cells of origin (US National institute of Environmental Health Sciences, 1989; Sell et al. 1987). Pronounced proliferation of oval cells is a frequent early event in hepatocarcinogenesis (Farber, 1956). There is a general agreement that oval cells may give rise to cholangiofibrotic lesions, which may lead to cholangiocellular tumors, but the possible relationship to hepatocellular tumors is remains unanswered (Steinberg et al. 1991).

2.3.1.1 Protocols in experimental hepatocarcinogenesis

Two types of protocols are used in experimental hepatocarcinogenesis studies (Bannasch et al. 1992). The first of the kind is the initiation-promotion protocol where as the second type is the initiation-selection protocol. (Bannasch et al. 1986).
2.3.1.1.1 The initiation-promotion protocol:
In the initiation-promotion protocol, a promoting agent such as phenobarbital is administered after the initiation with a known initiating agent. Partial hepatectomy is performed before the administration of the initiator. Partial hepatectomy induced rapid proliferation of hepatocytes is an important requisite for the administration of the initiator. The protocol can be used for screening initiating or promoting agents. In case of screening an initiating agent, the promoter is a standard agent, and when screening a promoting agent the initiator used is a standard.

2.3.1.1.2 The initiation-selection protocol:
The initiation-selection protocol utilizes the fact that the metabolites of 2-AAF being mitotic inhibitors, there is a selective inhibition of the growth of the normal hepatocytes where AAF is metabolised, but not in altered hepatocytes as they show suppressed Cyt P450 enzymes. Ito and colleagues have developed the medium term assays on the basis of the above phenomenon originally discovered by Farber (1976) for testing chemical carcinogens and modifiers of hepatocarcinogenesis (Tsuda et al. 1984; Ito et al. 1989). An obvious advantage of the initiation-selection protocols is the very rapid production of large number of focal and nodular hepatic lesions. Using these approaches Ito and colleagues were able to study induction of foci of altered hepatocytes (FAH) by 140 chemicals and observed a good correlation between their results and reported carcinogenic activities in long term bioassays (Ito et al. 1988). The pre-neoplastic nature of FAH has been inferred mainly from extensive investigations on the process of hepatocarcinogenesis induced by chemicals in rats (Bannasch, 1968; Emmelot and Scherer, 1980; Williams, 1980). The results of Ito et al (1988) suggest that irrespective of any phenotypic reversion, any production of an increased incidence of FAH and nodular liver lesions might be sufficient to indicate a carcinogenic effect on the liver (Bannasch, 1986).
2.3.2 Standard models for short term hepatocarcinogenicity assays

2.3.2.1 The Solt and Farber model (1976)

In this system preneoplastic basophilic foci can be detected within four weeks when a single dose of N-nitrosodiethylamine (NDEA/DEN) is followed by administration of AAF (0.02% in diet) for two weeks coupled with partial hepatectomy. AAF treatment exerts a selective growth advantage on the DEN initiated cells over that of the normal hepatocytes and the partial hepatectomy functions as a generalized growth stimulus. In this model the initiating activity of a chemical can be studied very well, DEN is a known carcinogen, and can be substituted with a test chemical whose initiating activity is to be known.

2.3.2.2 The Tsuda model (1980)

It is slight variation of the Solt and Farber model. Here a single dose of the test compound is given to rats 12-18 hrs after partial hepatectomy followed by feeding of AAF for 2-4 weeks, combined with the administration of a single toxic dose of carbon tetrachloride administered at week 3. The animals are killed at week 5 and the enzyme-altered foci are quantified. This model can be used to study the initiating activity of a test chemical in a similar manner as in the above method.

2.3.2.3 Hasegawa et al. model (1982)

This method can be used to detect the initiating potential the test compound. The test chemical is fed for 6 weeks with partial hepatectomy at week 1. From the 7th week AAF is administered for the 2 weeks 7th and 8th with one toxic dose of carbon tetrachloride. Carcinogens that usually produce tumors in extra-hepatic organs can be screened by this method.

2.3.2.4 Pitot et al. (1978)

DEN (low dose) is administered within 24 hours of 2/3 partial hepatectomy. Upto 8 weeks later the rats are administered phenobarbital (0.05%) in water or a known promoter for the next 12-24 weeks. This model can be used for screening initiating and promoting agents.
2.3.2.5 The Ito model (1980)

The protocol was designed to identify agents with tumor promoting activity. Following a high dose of a known standard initiator e.g. DEN (200mg/kg i.p) the promoting agent (test chemical) is administered after a 2-week rest period. A week after this, 2/3 partial hepatectomy is performed and the promoting agent administration is continued for next 5 weeks after which the animal is sacrificed and enzyme altered foci are detected and quantified.

2.3.2.6 Numoto et al. (1984)

This model was also proposed to identify promoting agents. Here after multiple treatments with 2-AAF for a period of 8 weeks, the test drug is administered since week 10 and continued to the 34th week. The enzyme-altered foci in the liver are detected and quantified.

2.3.3 Distinction between different types of pre-neoplastic lesions

The phenotypic patterns of pre-neoplasia appear to be varied as those of neoplasia, each histologically and cytologically defined tumor type presenting its own histo- and cytogenesis. Friedrich-Freska (1969) and his associates first described the enzyme-altered focus or liver island. These lesions, which developed long before carcinomas appeared, were characterized by small foci of cells that were devoid of glucose-6-phosphatase activity. Rabes (1972) later demonstrated that these foci could also be identified by their deficiency of canalicular ATPase activity.

Bannasch was able to distinguish with light microscopy at least 4 different types of altered hepatocytes: clear glycogen storage cells, acidophilic glycogen storage cells, fat storage cells, and basophilic cells which were poor in glycogen and rich in ribosomes. In contrast to changes in dysplastic and neoplastic cell populations, which conventionally are mainly defined on the basis of nuclear alterations, the phenotypic cellular changes related to pre-neoplasia pertain predominantly to the cytoplasm and fall into four main categories (Bannasch, 1988):

1. Alterations in the expression of enzymes, particularly those involved in carbohydrate and drug metabolism.
2. Changes in the content of metabolites, especially excessive storage of macromolecules such as glycogen, glycosaminoglycans, ribonucleoproteins and lipids.

3. Alterations in the organization of organelles, such as mitochondria, peroxisomes or the endoplasmic reticulum.

4. Increased cell proliferation and nuclear alterations.

2.3.3.1 Histochemical markers for preneoplastic lesions

Various histochemical and immunohistochemical techniques are available to distinguish the altered hepatocytes from the normal hepatocytes (Pitot and Sirica, 1980).

1. Altered metabolism of glycogen, retention of glycogen on fasting.

2. Deficient in the enzyme activities of:

- Glucose-6-phosphatase
- Adenosine triphosphatase (canalicular)
- β-glucuronidase
- Serine dehydratase
- Acid phosphatase
- Glycogen phosphorylase
- Ribonucleases and deoxyribonucleases

3. Exhibit increased activity of

- Fetal hepatocyte enzyme γ-glutamyl transpeptidase.
- Arylesterase
- Epoxide hydratase
- DT-diaphorase
- β-subunit of chorionic gonadotropin.
- α-Fetoprotein

4. Express preneoplastic antigen (Glutathions S-transferase placental form)

5. Show a deficiency to store iron.

6. Resistant to the cytotoxic action of hepatotoxins and carcinogens.

7. Exhibit an elevated DNA synthesis and mitosis.
2.3.4 Factors effecting the process of hepatocarcinogenesis

There are several factors that may modify the outcome of carcinogenesis. These modifiers may be exogenous (dietary) or endogenous (hormonal) factors. Thyroidectomy and adrenalectomy completely inhibit the production of hepatoma by 2-AAF in male rats. Testosterone administration to young male castrated rats causes a marked increase in the incidence of hepatic neoplasms in rats' fed 2-AAF than the rats receiving only the carcinogen. The sex of the animal also affects the process. Male rats are more susceptible to the hepatocarcinogenic action of 2-AAF than the female rats. Ovariectomy and choline deficient diets are reported to enhance the tumor promotion by phenobarbital (Hendrich et al. 1992; Shinozuka and Lombardi, 1980).

2.3.5 The Reversibility of Focal Lesions

The FAH are considered to be reversible in nature. Druckrey (1967) suggested a stop protocol in which a carcinogenic compound is administered for a limited period (3-7 weeks) and the animals investigated from 4 weeks to several months after the withdrawal (Bannasch and Zerban, 1992). The advantage of this approach is that evaluation of FAH is not complicated by reversion linked phenotypic instabilities, since they are largely excluded after 4 weeks withdrawal of the compound.

2.3.6 Types of FAH:

Different types of FAH have been distinguished in rats in detailed light and electron microscopical studies on carcinogen-induced changes in cytoplasmic components of the hepatocytes, such as glycogen, the endoplasmic reticulum, ribosomes and peroxisomes (Squire and Levitt, 1975; Maronpot et al. 1986). They are divided into three basic types depending on their morphology and staining characteristics:

2.3.6.1 Clear cell focus: These are usually small and represent the earliest types of foci of altered hepatocytes (FAH), which emerge in a dose and time dependent manner during hepatocarcinogenesis induced in rats with chemical carcinogens (Moore et al, 1982; Bannasch et al. 1989). These
are the glycogen storage foci observed on staining with the periodic acid-Schiff’s reagent for glycogen. They are located in the peripheral or intermediate parts of the liver lobule but on continued administration may move over to the centrilocular regions (Bannasch 1968).

2.3.6.2 Acidophilic cell foci: These are also known as ‘eosinophilic’ (Stewart et al., 1980), when the sections are stained with haematoxylin-eosin. They are closely related to, and are often combined with, glycogenolytic clear cell foci. They show a pronounced proliferation of the smooth endoplasmic reticulum, which is associated with the excessive storage of glycogen (Bannasch et al., 1985).

2.3.6.3 Basophilic cell foci: Foci exhibiting exclusively basophilic characteristics and loss of glycogen are widely accepted for decades as pre-stages of hepatocellular carcinomas. The diffusely basophilic foci poor in glycogen represent a late type of FAH, resulting from sequential cellular changes which lead from clear and acidophilic foci that store glycogen in excess through different stages to the basophilic cell population that prevails in hepatocellular carcinomas (Enzmann and Bannasch, 1987).

2.3.6.4 Another type of a focus distinguished by its morphological characteristics is observed.

Tigroid-cell focus: The cells of the tigroid-cell focus are usually small and are characterized by abundant basophilic bodies (rough endoplasmic reticulum), which are arranged in long bands with a stripy (tigroid) pattern. Apart from the biochemical phenotype the tigroid cell foci also differ in their potential to progress to a neoplasm. In aflatoxin-treated Sprague-Dawley rats during progression the tigroid pattern frequently changed to diffuse cytoplasmic basophilia. The tigroid cell foci can be classified as preneoplastic lesions with a low potential for neoplastic progression (Bannasch and Zerban, 1990). In mice the, potential for these basophilic foci to progress to both adenomas and carcinomas has
been well documented. (Goldfarb et al. 1983; Barnasch and Zebran, 1992)

2.3.7 Quantification of FAH

In the 19th century French geologist Delesse and the English microscopist Henry Sorby independently enunciated the principle that on average the fractional area of a particular sort of component on sections taken of a solid body, is directly proportional to the fractional volume of that component in the original solid body. Later Saltykov (1967) and others provided formulae and methods using which the numerical density, surface density, etc. of 3D objects of different sizes and shapes could be derived accurately from 2-dimensional transections in the containing samples. The derivation of such three-dimensional information from a two-dimensional image is known as 'Stereology' which is based on specialized field of mathematics called 'probabilistic geometry'. The valuable and reliable information provided by 'quantitative stereology' has earned the field an important place in the fields of geology, anatomy, metallography, electron microscopy and others (Hendrich et al. 1987).

The number of foci/liver (= numerical density) bears a direct relationship with the number of cells initiated and so provides information on the initiation potency of the substance whereas the volume of the foci expressed either per unit volume, total volume or as % of liver is related to the promotion potential of the substance since the volume is an index of the growth of the initiated cells under the influence of the promoter. Data of both categories provide valuable information about the development of FAH and their relation to carcinogenesis (Ito et al., 1989). All stereological approaches start with the assumption that FAH are spherical. This was well documented in one experimental model for small foci by three-dimensional reconstruction of focal transections (Pugh et al. 1983). Imaida et al. (1989) showed that larger FAH might have a more complex shape. Various workers have tried the stereological approach and finally conclude that all of these approaches adequately represent the modifying potential of several chemicals on the
development of GST-P/enzyme altered positive foci. The stereological approaches appear to be superior to other reported methods and the assumption that (small) FAH are spherical may be considered a useful approximation (Pitot et al. 1989).
2.4 Dapsone:

2.4.1 Nomenclature:

CAS Reg. No.: 80-08-0.

CAS Name: Benzamine, 4,4'-sulfonylbis.

IUPAC Systematic Name: Bis(4-aminophenyl)sulfone.

Synonyms: Bis(4 aminophenyl) sulfone; bis(paraaminophenyl) sulfone; diamino-4,4'- diphenyl sulfone; 4,4'-diaminodiphenyl sulfone; para,para- diaminodiphenyl sulfone; di(4-aminophenyl) sulfone; di(para-amino phenyl)sulfone; diphenylsulfin; diphenylsulphone; diphenylsulphon; 1,1'- sulfonyl(4-aminobenzene); 4,4' sulfonylbisaniline; 4,4' sulfonylbisbenzamine. Para,para-sulfonylbisbenzenamine; 4,4' sulfonyldianiline; para,para sulfonyldianiline; 1,1'-sulfonylbis(4-aminobenzene).

2.4.1 Brief history of dapsone

Fromm and Wittmann first synthesized dapsone (DDS) in 1908. The discovery was not based on the search for a solution of a therapeutic problem but represented an achievement in the field of chemistry aimed at identifying a molecule for producing azo dyes. Unrelated to Fromm and Wittmann's discovery, intensive efforts were being made at that time to combat bacterial infections by means of chemotherapy and many sulfonamide compounds were tested. In 1933, the first effective sulfonamide compound was submitted to clinical trials and it was put on the market as Prontosil in 1935. This represented a great achievement of Gerhard Domagk who discovered that certain sulfonamides that had been developed by the chemists with whom he collaborated, Drs. Klarer and Mietzsch, showed antibacterial potential (Wolf et al. 2002). DDS was first marketed in the USA in 1957 (Windolz, 1976). It was used primarily as an anti-infective in the prophylaxis of malaria and then in the treatment of leprosy.

2.4.2 Therapeutic uses of dapsone

DDS has high activity against Mycobacterium leprae and is the drug of choice for all forms of leprosy. It is also used in the treatment of relapsing polychondritis and in the prophylaxis of malaria. DDS has also found uses in
the treatment of tuberculosis and dermatitis hepatiformis. Since the early 1950s, DDS was recognized as being uniquely effective against a number of non-infectious inflammatory diseases, of which dermatitis herpetiformis is the best known (Bernstein and Lorincz, 1981). Recently DDS has been used in the treatment of AIDS related infections like pneumocystis carinii pneumonia and in the prophylaxis of toxoplasmosis (Behbahani et al., 1995). It is also used in a number of inflammatory disorders. Various reports have demonstrated that DDS can produce improvement in rheumatoid arthritis. Other skin disorders that have responded to DDS include erythema alavetum dilutinum, miliary lupus, pemphigoid and pemphigus, psoriasis, pyoderma gangrenosum and sweets syndrome. DDS has also been useful in brown spider bites. DDS is also used in veterinary practice against coccidiosis in cattle. A considerable number of other inflammatory as well as bullous diseases have been shown to respond in varying degrees to DDS, although the drug is not approved for all of them.

2.4.3 Mechanism of action:

The sulfones are chemically related to sulfonamides. Although many sulfones have been synthesized, DDS still remains the drug of choice for leprosy. DDS acts against bacteria and protozoa in the same way as sulfonamides, that is by inhibiting the synthesis of dihydrofolic acid through competition with para-amino-benzoate for the active site of dihydropteroate synthetase.

The anti-inflammatory action of the drug is unrelated to its antibacterial action and is still not fully understood. An inflammatory disease, which responds to DDS, is almost invariably associated with the infiltration of large numbers of polymorphonuclear leukocytes into the affected tissue. Various mechanisms affecting the function of neutrophils have been proposed. Perhaps the best-studied action of DDS is its interference with the myeloperoxidase-hydrogen peroxide-halide mediated cytotoxic system in neutrophils (Lang, 1979).
2.4.4 Pharmacokinetics

DDS is absorbed rapidly and nearly completely from the gastrointestinal tract and the oral bioavailability is about 70 – 80%. An acidic environment is required for its absorption. DDS is distributed throughout total body water and is present in all tissues.

Peak concentrations of DDS in plasma are reached within 2 to 8 hours after administration. The mean half-life of elimination is about 20 to 30 hours. Twenty-four hours after oral ingestion of 100 mg, plasma concentrations range from 0.4 to 1.2 μg/ml. A dose of 100 mg per day produces steady-state plasma concentrations of free DDS of 2 to 6 μmol/L. About 70% of the drug is bound to plasma proteins (Zuidema et al. 1986; Ellard, 1966). It tends to be retained in skin and muscle and especially in the liver and kidney, traces of the drug are present in these organs up to 3 weeks after cessation of therapy.

2.4.5 Metabolism

After absorption from the gastrointestinal tract, DDS is transported through the portal circulation to the liver and is metabolized by two distinct routes, N-acetylation and N-hydroxylation. DDS is acetylated by N-Acetyl transferase in the liver to its major metabolite, monoacetyl DDS (MADDS). MADDS is again deacetylated to DDS and a equilibrium is established in a few hours. It is also N-hydroxylated to DDS hydroxylamine in the liver by the mixed function oxidase system in the presence of oxygen and NADPH. Both the metabolites have a very low activity. Mitra et al. (1995) have reported that the CYP<sub>450</sub> accounting for the majority of DDS hydroxylamine formation in vivo is the CYP2E1. To a lesser extent, CYP2C also metabolizes DDS.

A single dose of disulfiram, a slowly reversing inhibitor of CYP2E1 in vivo, inhibited DDS hydroxylamine formation clearance by 73%, and inhibited methemoglobin formation by 78% in a group of healthy volunteers. DDS-NHY has been reported to be catalyzed by cytochrome P<sub>450</sub> (CYP) 2C9, CYP3A4, and CYP2E1 (Gill et al. 1995). The involvement of constitutive enzymes CYP2C6 and/or CYP2C11, as well as CYP3A1 (non-constitutive) in the metabolism of DDS have also been reported (Vage and Svensson, 1994).
Winter et al. (2000) reported 45% decrease in N-hydroxyl metabolites of DDS when human liver microsomes pretreated with tolbutamide were treated with clinical concentrations of DDS suggesting the isoforms CYP2C8/9 are involved in the conversion of DDS to its hydroxylamine. Disulfiram an \textit{in-vivo} CYP2E1 inhibitor produced a 65% decrease in DDS-NHY in non-HIV infected subjects. It is also observed that fluconazole a CYP2C and CYP3A4 inhibitor that does not inhibit CYP2E1 inhibited DDS-NHY by 50%.

\subsection*{2.4.6 Dapsone toxicity}

In particular methemoglobin formation is putatively initiated by N-oxidation, resulting in the formation of a hydroxylamine metabolite by cytochrome P$_{450}$ (Ellard et al. 1972). Methemoglobin levels under 20% are not usually associated with symptoms. Dyspnea, nausea and tachycardia usually occur at levels of 30% or above, while lethargy, stupor and deteriorating consciousness occur as methemoglobin levels approach 55%. Levels of 70% are usually fatal leading to cyanosis and loss of consciousness and coma (Coleman and Coleman, 1996). Motor neuropathy is the most common neurological effect of DDS in-patients other than leprosy. The symptoms include weakness of hands and limbs and paraesthesia of digits. The sensory impairment is uncommon.

In male rats, the i.p. LD$_{50}$ is about 200mg/kg and the oral LD$_{50}$ is about 630mg/kg; in female rats the oral LD$_{50}$ is about 650mg/kg; the i.p. LD$_{50}$ in male mice is about 230mg/kg (Wu and DuBois, 1970). The half-life of dapsone in rats is about 6 hours (Murray, et al. 1972).

\subsection*{2.4.7 Methaemoglobinemia}

Methaemoglobin is an abnormal form of hemoglobin with a reduced capacity of binding with oxygen. Methaemoglobin is produced when the ferrous (Fe$^{2+}$) iron within the heme molecule is oxidized to the ferric (Fe$^{3+}$) state. In the oxidized state, the ferric iron within the heme subunit is incapable of binding oxygen, because of a stoichiometric alteration of the molecule. In addition to decreasing the amount of oxygen the hemoglobin can transport, the
presence of an oxidized heme unit within the molecule also decreases the ability of the reduced heme subunits to offload oxygen to the tissues.

The constant presence of endogenous and exogenous oxidizing substances results in the continuous formation of methaemoglobin. In normal individuals, the methaemoglobin level is maintained below 1% through two metabolic pathways. The major pathway involves the enzymatic reduction of the glycolytic product NAD (nicotinamide adenine-dinucleotide) to NADH (nicotinamide adenine-dinucleotide reduced). NADH then acts as an electron donor in the reduction of the ferric (Fe^{3+}) iron of the methaemoglobin to ferrous (Fe^{2+}). The enzyme NADH methaemoglobin reductase is required for the reduction of NAD and methaemoglobin (Rasbridge and Scott, 1973).

Within the erythrocyte, the enzymatic reduction of methaemoglobin may also be accomplished through the reduction of NADP (nicotinamide adenine-dinucleotide phosphate) that is produced via the hexose monophosphate shunt. NADPH (nicotinamide adenine-dinucleotide phosphate reduced) in turn can be used to reduce oxidized glutathione to reduce glutathione (GSH). Therefore, individuals having a deficient glucose-6-phosphate dehydrogenase are prone to methemoglobinemia (Frischer and Ahmed, 1987).

DDS hydroxylamine reacts with oxyhemoglobin (Fe^{2+}) to form methemoglobin (Fe^{3+}) and nitrosoarene, which, in turn, is reduced to hydroxylamine by either NADPH methemoglobin reductase or glutathione. The hydroxylamine then reacts with another molecule of oxyhemoglobin, thus continuing the redox cycle. Each hydroxylamine molecule is capable of oxidizing up to five oxyhemoglobin molecules and the cycle only ceases when the erythrocyte is almost totally depleted of glutathione (Kramer, et al. 1972).

The fate of the toxic metabolite of DDS, DDS hydroxylamine, has been studied in the human red cell. The parent amine was produced from DDS hydroxylamine during methemoglobin formation in the red cells, and there was a linear relationship between hydroxylamine-dependent methemoglobin formation and conversion of hydroxylamine to DDS. A cycle exists between the hepatic oxidation of DDS to its hydroxylamine form and reduction to amine.
within the red cell, a process, which might lead to re-oxidation by the hepatic cytochrome P450. They speculated that this process might contribute to the persistence of the drug in vivo (Coleman and Jacobus, 1993).

Methemoglobinemia occurs to some extent in all patients receiving DDS and becomes less pronounced as treatment is continued due to an adaptive increase in the activity of NADH-dependent reductase in the erythrocytes. Mahmud, et al. (1997) has reported that DDS does not oxidize haemoglobin in the absence of NADPH. In the presence of human liver microsomes, the toxicity of DDS analogues was generally reduced compared with the toxicity observed in the presence of rat microsomes. The authors tested several amines and in the presence of liver enzymes DDS was the most potent methaemoglobin former. The sulfone group is responsible for the toxic effects and is also necessary for the therapeutic effects of DDS. 4,4'-Diaminodiphenyl ether (DDE) and 4,4'-diaminodiphenyl thioether (DDT) do not produce methaemoglobin in-vitro or in-vivo and reflects a low rate of metabolism of these compounds (Coleman, et al. 1991) and both these compounds are hepatocarcinogens in the rat (Ashby and Tennant, 1991).

Some agents have direct oxidizing effects on the hemoglobin while other agents cause the formation of oxygen and peroxide free radicals, which are able to oxidize hemoglobin. A pathway showing formation of methaemoglobin is shown in Fig. 2.

DDS -NOH mediated oxidation of hemoglobin outstrips the capacity of NADH methaemoglobin reductase to convert methaemoglobin back to hemoglobin (Griffith, 1980). DDS-NOH causes an oxidative stress on the erythrocytes, and due to the continuous oxidation - reduction reactions that take place in between hydroxylamine and the oxyhemoglobin molecule, hydronitroxide radical and hydrogen peroxide molecules may be formed as intermediates (Stolze and Nohl, 1989; Stolze, et al. 1996). Hydrogen peroxide generates other active oxygen species, which, together with reactive hydroxylamine products, are capable of causing cellular damage.
Free radicals or reactive groups causes oxidation of GSH to form Glu-Hb mixed disulphides

Sulphonyl group and hydroxyl amine (highly reactive) (DDS-NOH + HbO₂ → [MHb(III)-OOH] + DDS-NO)

GSH → glutathione-Hb mixed disulphides (GSSG)

Converted back to GSH by NADP reduced from hexose mono Phosphate shunt by G6PD

Fig. 2 Pathway for methemoglobin formation
2.4.8 Role of glutathione in methaemoglobinaemia

Glutathione (γ-glutamylcysteinylglycine; GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water-soluble is found mainly in the cell cytosol and other aqueous phases of the living system. It exists in two forms, the antioxidant "reduced glutathione" tripeptide abbreviated GSH and the oxidized form, a sulfur-sulfur linked compound, known as glutathione disulfide or GSSG.

GSH has potent electron-donating capacity. Its high redox potential renders GSH both a potent antioxidant and a convenient cofactor for enzymatic reactions that require readily available electron pairs. Glutathione is present inside cells mainly in its reduced (electron-rich, antioxidant) GSH form. It is an essential cofactor for antioxidant enzymes, namely the GSH peroxidases. After GSH has been oxidized to GSSG, the recycling of GSSG to GSH is accomplished mainly by the enzyme glutathione reductase. This enzyme uses as its source of electrons the coenzyme NADPH (nicotinamide adenine dinucleotide phosphate, reduced). Therefore NADPH, coming mainly from the pentose phosphate shunt, is the predominant source of GSH reducing power.

The relation between DDS administration and glutathione level is significant because DDS hydroxylamine is reported to induce a rapid and concentration-dependent loss of erythrocytic reduced- glutathione content with a concomitant increase in protein-glutathione mixed disulfide formation in both human and rat red cell suspensions (Glader and Cornad, 1973). In the presence of glutathione peroxidase, GSH can combine with oxidant compounds capable of changing hemoglobin to methaemoglobin. Glutathione is oxidized during treatment with hydroxylamine and is mainly converted to glutathione hemoglobin mixed disulfides. This formation of glutathione-protein disulphides acts as a store of oxidized glutathione. Through formation of glutathione-protein mixed disulfides, erythrocytes are easily and quickly able to restore their glutathione levels under recovered conditions (Kosower, et al. 1982).
Through its significant reducing power, GSH also makes major contributions to the recycling of other antioxidants that have become oxidized. GSH plays a role in such diverse biological processes as protein synthesis, enzyme catalysis, transmembrane transport, receptor action, intermediary metabolism, and cell maturation. Glutathione's reducing power is used in conjunction with ascorbate and other antioxidants to protect the entire spectrum of bio-molecules, to help regulate their function, and to facilitate the survival and optimal performance of the cell as a living unit.

The liver is the organ most involved with the detoxification of xenobiotics, and also is the main storage locale for GSH (actually exporting GSH to the other organs). Glutathione reaches its highest intracellular concentrations (about 10mM) in the parenchymal cells ("hepatocytes") of the healthy liver. The hepatocytes are highly specialized to synthesize GSH from its precursors or to recycle it from GSSG, as well as to utilize GSH against potential toxicants.

2.4.9 Contra-indications and precautions:

DDS is considered unsafe and should not be used in the following conditions: severe anemia, porphyria, deficiency of glucose-6-phosphate dehydrogenase, glutathione reductase or methemoglobin reductase, allergy to sulfonamides, or significant liver disease. It should not be paired with other hemolytics or dideoxyinosine. Before starting therapy, a complete blood count, reticulocyte count, glucose-6-phosphate dehydrogenase level, liver function studies, urinanalysis and renal function tests should be performed. During therapy, complete blood count, reticulocyte count, platelet count and leukocyte count with differential should be obtained weekly for the first month, then twice per month during the next two months and every three months thereafter. Liver and renal function should be tested every three months. Methemoglobin levels should be obtained in-patients who become symptomatic for methemoglobinemia.

Besides causing methaemoglobinemia, DDS may also cause hemolysis and the rbc's may contain Heinz bodies. The risk factors include glucose 6-
phosphate dehydrogenase deficiency, methaemoglobin reductase deficiency and hemoglobin M trait. Aplastic anemia, agranulocytosis, hypoalbuminaemia have also been reported. The other effects include nausea, vomiting, abdominal pain and anorexia. The CNS reactions include psychosis, headache, insomnia, vertigo and paraesthesia.

2.4.10 Carcinogenicity of Dapsone

Dapsone is structurally related to potent carcinogenic aromatic amines and it is reported that tumors occurred more frequently in-patients of leprosy (Fukushi and Sasaki, 1973) and dermatitis herpetiformis (Mansson, 1971) undergoing DDS therapy. It was first reported in 1973 by Bergel that DDS was weakly carcinogenic in male rats and was subsequently confirmed by the reports from the National Cancer Institute (National Cancer Institute, 1977) and the International Agency for Cancer Research. The principal tumors observed in these studies were mesenchymal tumors of abdominal organs, especially the spleen, (Goodman, et al. 1984) and an increase in the incidence of thyroid tumors was also observed (Griciute and Tomatis, 1980). The reports have thus caused concern that DDS may present a risk to patients receiving DDS treatment for leprosy (Bloom, 1979). Although the dose ranged from 42 – 300 mg/kg/day for the lifetime of the animals, whereas the usual maximum dose of DDS for humans is 100 mg/day or about 1 – 2 mg/kg/day.

Two derivatives of DDS 4,4'-diaminodiphenyl sulfide (DDSD) and 4,4'-diaminodiphenyl sulfoxide (DDSO) were mutagenic in the AMES test, but it is also noteworthy that these two derivatives were not detected in the urine samples of patients who had consumed DDS (Peters, et al. 1983). DDS also failed to induce sister chromatid exchanges or chromosomal aberrations in mice (Dr. P.S.R. Murthy, unpublished observations). In vivo studies conducted on DDS have shown it to cause pulmonary neoplasms in the mouse (Peraino, et al. 1978). On the basis of available animal data, the International Agency for Research on Cancer (IARC, 1987) concludes that the animal studies conducted so far provide only a limited evidence for the carcinogenic potential of DDS.
2.4.11 Modulators of DDS metabolism

DDS is primarily metabolized by the CYP_{450} microsomal enzyme system in the liver. It has been established that DDS is a substrate for CYP3A4, CYP2C9 Cytochrome P_{450} isoforms. And agents that are known to inhibit or activate this system do interfere with DDS metabolism. Cimetidine, disulfiram, sulfaphenazole, tolbutamide, ketoconazole are known to inhibit CYP3A4, CYP2C8/9 and CYP2C11. DDS itself is known to activate CYP3A and thus stimulating its metabolism.

2.4.11.1 Cimetidine

Cimetidine, ranitidine, famotidine and nizatidine are H₂-receptor antagonists and are used in the treatment of peptic ulcer. These drugs act by inhibiting competitively the interaction of histamine with H₂ receptor (Brogden, et al. 1982). Chemically cimetidine is 2 N'-cyano- N-methyl- N'-[2-[(5-methyl-1 H-imidazol-4-yl)methyl]thio]-ethyl, guanidine. Cimetidine thus blocks the peptic acid secretion via stimulation of histaminergic H₂ receptors. Cimetidine also inhibits gastric acid secretion stimulated by food, histamine, pentagastrin, caffeine and insulin and also inhibits both daytime and nocturnal basal gastric acid secretion.

Cimetidine is rapidly absorbed after oral administration and peak levels occur in 45 to 90 minutes. The half-life of cimetidine is approximately two hours. In a 24-month toxicity study conducted in rats, at dose levels of 150, 378 and 950 mg/kg/day (approximately 8 to 48 times the recommended human dose), there was a dose related increase in the incidence of benign Leydig cell tumors in the higher dose group; there were no differences between the rats receiving 150 mg/kg/day and the untreated controls. These tumors were common in control groups as well as treated groups and the difference became apparent only in aged rats. The metabolite of cimetidine, nitrosocimetidine is known to be genotoxic, but has not been reported to produce tumors in rats or mice (Habs, et al. 1982; Anderson, et al. 1985).

Cimetidine, apparently through an effect on certain microsomal enzyme systems, has been reported to reduce the hepatic metabolism of warfarin-type
anticoagulants, phenytoin, propranolol, nifedipine, chlordiazepoxide, diazepam, certain tricyclic antidepressants, lidocaine, DDS, theophylline and metronidazole, thereby delaying elimination and increasing blood levels of these drugs (Lawrence, 1990).

Coleman, et al. (1990) had shown that the prior administration of cimetidine to DDS treated subjects reduce methaemoglobin levels by 60% with concomitant decrease in DDS- hydroxylamine metabolite of DDS. Administration of cimetidine diminished DDS-induced methemoglobin formation by 26% in dermatitis herpetiformis patients. As evidenced in their study, the authors speculated that the treatment-limiting toxicities of DDS might be diminished by co-administration of a suitable inhibitor of hydroxylamine formation, such as cimetidine (Coleman, et al. 1992).

2.4.11.2 Tolbutamide:

Actohexamide, Tolazamide, Chlorpropamide and Tolbutamide belong to the Sulfonylurea class of hypoglycemics, used in the treatment of Type II diabetes mellitus. Sulfonylurea’s are known to stimulate the insulin secretion by stimulating the receptors on pancreatic β-cells. Sulfonylurea’s also increase the sensitivity of peripheral tissues to insulin (Gaines, et al. 1988; Pfeifer, et al. 1981).

The major metabolite of tolbutamide is hydroxytolbutamide, with minor amounts of carboxytolbutamide. The major adverse effect of tolbutamide is hypoglycemia, and may be potentiated by sulfonamides, clofibrate, dicumarol, salicylates and phenylbutazone. The other effects include cholestatic jaundice, agranulocytosis, aplastic-hemolytic anemias, and generalized hypersensitivity reactions (Kahn and Shechter, 1990).

CYP2C8/9 isoform of CYP<sub>450</sub> are inhibited by tolbutamide and it has been reported that tolbutamide inhibits formation of DDS-NHY by 41% in vitro (Winter, et al. 2000).

2.5 The role of Glutathione in Carcinogenesis

Glutathione is mutagenic to bacteria and genotoxic to mammalian cells (Avishay, et al. 1993). GSH mutagenicity depends largely on the activity of γ-
glutamyl transpeptidase (GGT) since the breakdown products of GSH are oxidative. Papilloma producing epidermal cells with tumorigenicity were converted to fully tumorigenic cells upon transfection with a functional GGT cDNA (Yashimi, et al. 1992). GSH mutagenicity depends largely on the activity of GGT and is oxidative in its nature, requires molecular oxygen, is enhanced by iron, and is inhibited by catalase, peroxidase, radical scavengers and chelators that do not permit facile redox reactions of iron. The importance of oxidative damage in hepatocarcinogenesis process is demonstrated by findings that some antioxidants decrease tumor promotion in liver (Rao and Reddy, 1987).

In hepatocarcinogenesis induced by peroxisome proliferators, preneoplastic foci and tumors are GGT negative (Rao, et al. 1986). The peroxisome proliferators can induce oxidative damage by GGT independent routes. Promotion by orotic acid, gonadal hormones, pyrrolizidine alkaloids and bile acids are some of the examples of the mechanisms of hepatic tumors that do not involve oxidative damage. Transition metals catalyze the catabolism of GSH by GGT leading to oxidative damage, which in turn leads to hepatotoxicity and carcinogenicity. Oxidative metabolism of extra-cellular GSH, initiated by high levels of GGT in preneoplastic foci, may produce oxidative damage which could lead to increased frequency of genetic alterations and/or tumor promoting effects. These effects would help move the preneoplastic cell further along the track from pre-neoplasia to neoplasia (Stark, 1991).

2.6 Natural Products and Cancer Chemoprevention

Prof. Micheal Sporn of Dartmouth Medical School has strongly argued that "obsession with curing advanced disease has blinded cancer researchers to the promise of prevention. The concept that people with cancer were healthy until a doctor told them that they've got an invasive lesion makes no sense at all. And only a few believers in cancer chemoprevention are trying to change the viewpoint."
Chemoprevention is the attempt to use natural and synthetic compounds to intervene in the early precancerous stages of carcinogenesis, before invasive disease begins. The idea behind such and intervention is simple. Certain foods, including many vegetables, fruits and grains offer protection against various cancers. Chemoprevention researchers try to find substances, either components of food or pharmaceuticals- that possesses this capacity. The basic difference in between chemopreventive and chemotherapeutic agents is that chemopreventive agents are used when the disease is actually not present, or at a stage not detectable by currently available methods while the chemotherapeutic agents are used to treat a cancer. Chemotherapeutic agents kill the cancer cells more than the healthy cells but still can be quite toxic.

Food is a source of most promising chemopreventive compounds. These naturally occurring phytochemicals include vitamins such as A (and its analogues), C and E, as well as compounds without nutritional value, such as indoles, isothiocyanates, dithiolthiones and organosulfur compounds. Dithiolthiones that are found in cruciferous vegetables such as cauliflower, broccoli and cabbage are potential chemopreventives. A synthetic dithiolthione, Oltipraz has been shown to inhibit the development of tumors of the lung, colon, mammary glands and bladder in laboratory animals. Genistein from soy and tea extracts containing epigallocatechin gallate have also been reported to be chemopreventives. Most of these compounds have anti-oxidant properties, but some such as the dithiolthiones and isothiocyanates act by activating liver enzymes that can detoxify liver carcinogens.

A number of clinical trials dealing with natural products have been carried out since 1983. Alpha-tocopherol, beta-carotene, the retinoid isotretinoin, combinations of vitamins and minerals (linxian) and tamoxifen have been included in the clinical trials. Alpha-tocopherol, isotretinoin and linxian have proved to be good chemopreventives (Greenwald, 1996). Flavanoids in turmeric, β-carotene, α-tocopherol, eugenol, chlorophyll and hydroxychavicol in betel leaf (Piper betel, Linn.), gallic acid in garlic, vitamin c, indoles, isothiocyanates, dithiolthiones and organosulfur compounds from

Chemoprevention offers several benefits over chemotherapy. The most important benefit offered is related to the toxic effects. Being a constituent of the diet these are almost devoid of any side effects, when taken in the proper doses. The other advantages are the cost effectiveness and convenience of administration. Considering these major benefits further studies are being taken up by the American Health foundation and the National Cancer Institute (NIH) to identify new, potent, safe and easy to administer chemopreventives and promote the strategy of chemoprevention in day to day life.
MATERIALS AND METHODS
3.1 Animals

All the studies were carried out in male or female Charles Foster (CF) rats of the CDRI-National Laboratory Animal Center colony adhering to the CDRI Animal Ethics Committee guidelines for humane treatment of animals. At the start of the studies the male rats were young adults of 165±15gm. body weight and the female rats were virgin and nulliparous weighing 165±15gm.

3.2 Chemicals

4,4'-diaminodiphenyl sulfone (Dapsone; DDS), 2-acetamidoflourene (AAF; 2-AAF), N-nitrosodiethylamine (DEN), cimetidine (CMT), L-γ-glutamyl 4-methoxy 2-naphthylamide, glycylglycine, Fast Blue BB, periodic acid, pararosaniline HCl and saponin, were obtained from Sigma Chemicals, St. Louis, USA; phenol reagent from Centron Research Laboratories, India; phenobarbitone (PB) was from May and Baker, England; and tolbutamide (TLB) was obtained from Hoechst (India) Ltd., Mumbai. Picroliv (PLV) powder was obtained from Dr. S. C. Sharma, Division of Process Development, Central Drug Research Institute, Lucknow. Lyophilized betel leaf powder (BTL) was kindly provided by Dr. N. K. Dubey, National Botanical Research Institute, Lucknow. GST-P antibody was from Calbiochem and other antibodies were from Sigma Immunochemicals. All other chemicals were reagent grade obtained from EMerck (India) and SD Fine chemicals, India.

3.3 Hepatocarcinogenicity models used

3.3.1 The Pitot model: This is essentially as described by Pitot et. al, 1978. Female Charles-Foster rats received N-nitrosodiethylamine (DEN) in a single oral dose of 10mg/kg within 24 hours of 2/3rd partial hepatectomy. After a 2-week rest period, the promoter and/or the test agent was administered p.o once daily for 12 weeks. At the end of the 14th week post DEN, the rats were sacrificed.
3.3.2 The Medium-term model of Ito et al., 1980: Male Charles-Foster rats received a single intraperitoneal (i.p.) administration of N-nitrosodiethylamine (DEN) (200mg/kg). After a 2-week rest period the promoter and/or the test agent was administered p.o; once daily for the next 6 weeks. At the end of the first week of promoter and/or the test agent administration, 2/3\(^{rd}\) partial hepatectomy was performed and at the end of the 8\(^{th}\) week post DEN the rats were sacrificed.

3.4 Body weight monitoring

Body weights of rats were taken on the day of the start of the experiment (initial) and weekly thereafter till the termination of the experiment. The gain/loss in body weight was calculated by subtracting the later weight from the initial weight. It was then averaged for every group.

3.5 Monitoring food consumption

Daily food consumption by the rats was measured and recorded by the following procedure. A measured excess of rat diet consisting of standard pellets (100gm) and Bengal gram (20gm) was placed everyday in each cage housing 4 rats of a treatment group. After 24 hrs the leftovers of the pellets and Bengal gram in the cage were collected and weighed to calculate the amount consumed by a rat per day. At the end of the week the average weekly consumption of food by the rats was calculated.

3.6 Cage side observations:

Included in these are the general behavior and activity of the animals, fur coat appearance, any abnormal discharges from nasal, oral, anal and vaginal orifices, consistency of faeces, injuries, etc. These observations were made daily at the time of dose administration, drawing of blood samples and just before sacrifice.

3.7 Technique of 2/3 partial hepatectomy in rats

Surgical 2/3\(^{rd}\) hepatectomy in rat was performed following the procedure described by Anderssen (1971).

Prior to surgery, food was withdrawn overnight but the drinking water contained 10% glucose. The rat was anesthetized with anesthetic ether and
placed on a sterile surgical board. Careful asepsis was maintained throughout
the surgery. Through a median-line incision, reaching 3 or 4cm posteriorly
from the xiphoid process of the sternum, the large median lobe of the liver,
with the left lateral lobe, was expelled out, securely ligated at the base of the
lobes by sterile coarse linen and then excised. In this way lobes of hepatic
parenchyma ranging in extent from 65 to 70 % of the total liver were removed,
leaving within the peritoneum the right lateral lobe and the small caudate or
spigelian, lobe. The abdomen was closed in two layers, the peritoneum and
abdominal muscles were closed in first layer and the integument in the second.
There was no special postoperative care, except in place of water the animals
had access to food and drinking water containing 20% dextrose for the first
day, after which the normal diet and water were provided.

3.8 Drug preparation and administration

All the vehicles and test/standard substance solutions or suspensions were
either made fresh or stored at 4°C as dictated by the stability of the agents.
Standard practices for ensuring the sterility of the solutions were followed.

1. Agar (Vehicle): 0.2% solution in triple distilled water (TDW) of
microbiology grade agar was used as a suspending agent for test and
standard agents.

2. N-nitrosodiethylamine (DEN): Solution of DEN was prepared in sterile
0.9%NaCl at 100mg/ml or 10mg/ml concentrations for Ito and Pitot models,
respectively, and administered i.p. at a dose of 200mg/kg in the Ito model
and 10mg/kg orally (p.o.) in the Pitot model.

3. Dapsone (DDS): A fine suspension of DDS was prepared in 0.2% agar and
administered p.o. daily once by a feeding canula at the doses required.

4. 2-acetamidofluorene (AAF): A fine suspension of AAF (8mg/ml) was
prepared in 0.2% agar and administered once p.o. daily at a 20mg/kg/day
dose.

5. Tolbutamide (TLB): A fine suspension of TLB (50mg/ml) was prepared in
0.2% agar and administered once p.o. daily 1 hour before DDS
administration at a dose of 50mg/kg/day.
6. Cimetidine (CMT): A fine suspension of CMT (50mg/ml) was prepared in 0.2% agar and administered once p.o. daily 1 hour before DDS administration at a dose of 50mg/kg/day.

7. Picroliv (PLV): A solution of PLV at a concentration of 8mg/ml was prepared in distilled water and administered p.o. once at a dose of 10mg/kg/day, 1 hour before DDS administration.

8. Betel leaf powder (BTL): 40mg/ml suspension of crude betel leaves powder was prepared in distilled water and administered p.o. at a dose of 200mg/kg/day, 1 hour before DDS, PB or AAF administration or before DEN administration as specified in the studies.

9. Phenobarbitone (PB): Phenobarbitone (18mg/ml) solution was prepared in distilled water and administered p.o. once at a dose of 90mg/kg/day.

Strict asepsis was maintained for the agents that were administered intraperitoneally.

3.9 Protocols for individual studies

1. PROMOTION OF DEN-INITIATED HEPATOCARCINOGENESIS BY DAPSONE (DDS)

1.1 Comparative study on the promotion efficacy of DDS and the standard promoters 2-acetamidoflourene (AAF) and phenobarbitone (PB) in the Ito model of hepatocarcinogenesis

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of N-nitrosodiethylamine (DEN) (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 4 groups. The animals belonging to the DEN-Vehicle group received once daily administration of only the agar solution (Vehicle) for 6 weeks. DEN-AAF group received once daily oral administration of AAF (20mg/kg/day) for 6 weeks. While the DEN-PB group received PB at a dose of 90mg/kg/day and the DEN-DDS group was administered DDS at a dose of 50mg/kg/day for 6 weeks. One week after the start of the agent/vehicle administration, surgical
partial hepatectomy was performed on all rats. At the end of the 8th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.

**Group 1: DEN-Vehicle (n=7)**

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**Group 2: DEN-DDS (n=6)**

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<td>DEN</td>
<td>DDS 50mg/kg (once daily)</td>
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**Group 3: DEN-PB (n=7)**

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<td>DEN</td>
<td>PB 90mg/kg (once daily)</td>
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**Group 4: DEN-AAF (n=10)**

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<td>DEN</td>
<td>AAF 20mg/kg (once daily)</td>
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1.2 Dose response study on DDS in the Pitot model of hepatocarcinogenicity

The study was conducted in Female Charles Foster rats. Within 24 hours of 2/3rd partial hepatectomy the rats were administered DEN (10mg/kg) p.o. After 2 weeks rest period, the rats were divided into 4 groups. The DEN-DDS10 group was administered DDS at a dose of 10mg/kg/day, once daily for 12 weeks. Similarly the DEN-DDS25 and DEN-DDS50 groups were administered DDS at doses 25mg/kg and 50 mg/kg/day for 12 weeks. The control group received the vehicle (0.2% agar) only in place of DDS. At the
end of the 14th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is shown below.

Group 5: DEN-DDS (n=5)

Group 6: DEN-DDS (n=5)

Group 7: DEN-DDS (n=13)

Group 8: DEN-Vehicle (n=5)
2. MODULATION OF DDS PROMOTED HEPATOCARCINOGENESIS

2.1 Modulation by Cytochrome P<sub>450</sub> enzyme inhibitors

2.1.1 Cimetidine (CMT; a CYP 3A4 inhibitor) and Tolbutamide (TLB; a CYP 2C9 inhibitor) on DDS Promoted Hepatocarcinogenesis in Pitot Model

The study was conducted in Female Charles Foster rats. Within 24 hours of 2/3rd partial hepatectomy surgery the rats were administered DEN (10mg/kg) p.o. After 2 weeks rest period, the rats were divided into 3 groups. The DEN-DDS group was administered DDS (50mg/kg/day; p.o.) for 12 weeks. The DEN-DDS-TLB and DEN-DDS-CMT groups were administered tolbutamide (TLB) and cimetidine (CMT), both at 50mg/kg/day dose, 1 hour before the administration of DDS, for 12 weeks. At the end of the 14<sup>th</sup> week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.

**Group 7: DEN-DDS (n=13)**

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**Group 9: DEN-DDS-TLB (n=8)**

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**Group 10: DEN-DDS-CMT (n=8)**

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2.1.2 Cimetidine (CMT; a CYP 3A4 inhibitor) and Tolbutamide (TLB; a CYP 2C9 inhibitor) on DDS Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 3 groups. The animals of DEN-DDS group received once daily oral administration of DDS (50mg/kg/day) for 6 weeks. The DEN-DDS-TLB and DEN-DDS-CMT groups received TLB (50mg/kg/day) and CMT (50mg/kg/day) respectively one hour before the administration of DDS for 6 weeks. One week after the start of the test agent and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.
2.1.3 Effect of TLB (a CYP 2C9 Inhibitor) on PB Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 2 groups. The animals of DEN-PB group received once daily oral administration of PB (90mg/kg/day) for 6 weeks. The DEN-PB-TLB group received a suspension of TLB (50mg/kg/day) one-hour before the administration of PB, for 6 weeks. One week after the start of the test agent and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN treatment the rats were sacrificed. A schematic representation of the treatment schedule is given below.

Group 3: DEN-PB (n=7)

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Group 3: DEN-PB-TLB (n=8)

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</table>
2.2 MODULATION BY NATURAL PRODUCTS

2.2.1 Effect of Picroliv (PLV) on DDS Promoted Hepatocarcinogenesis in Pitot Model

The study was conducted in Female Charles Foster rats. Within 24 hours of 2/3\textsuperscript{rd} partial hepatectomy surgery the rats were administered DEN (10mg/kg) p.o. After 2 weeks rest period, the rats were divided into 2 groups. The DEN-DDS group was administered DDS (50mg/kg/day) for 12 weeks. The DEN-DDS-PLV group was administered Picroliv (PLV) 10mg/kg/day one hour before the administration of DDS for 12 weeks. At the end of the 14\textsuperscript{th} week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.

**Group 7: DEN-DDS (n=13)**

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<th>Week</th>
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<td>DEN</td>
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<td>DDS50mg/kg (once daily)</td>
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**Group 14: DEN-DDS-PLV (n=8)**

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<td>DDS50mg/kg + PLV 10mg/kg (once daily)</td>
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<td>PH</td>
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2.2.2 Effect of PLV on DDS Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 2 groups. The animals of DEN-DDS group received once daily oral administration of DDS (50mg/kg/day) for 6 weeks. The DEN-DDS-PLV group received suspension of PLV (10mg/kg/day) one hour before the administration of DDS, for 6 weeks. One week after the start of the test agent and/or promoter administration, surgical partial
hepatectomy was performed on all rats. At the end of the 8th week post DEN treatment the rats were sacrificed. A schematic representation of the treatment schedule is given below.

**Group 2: DEN-DDS (n=6)**

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<tbody>
<tr>
<td>Den</td>
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<td>DEN</td>
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<td>DDS 50 mg/kg (once daily)</td>
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**Group 3: DEN-DDS-PLV (n=5)**

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<tr>
<td>DDS 50 mg/kg + PLV 10 mg/kg (once daily)</td>
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2.2.3 Effect of Betel Leaf (BTL) on DDS Promoted Hepatocarcinogenesis in Pitot Model

The study was conducted in Female Charles Foster rats. Within 24 hours of 2/3rd partial hepatectomy surgery the rats were administered DEN (10 mg/kg) p.o. After 2 weeks rest period, the rats were divided into 2 groups. The DEN-DDS group was administered DDS (50 mg/kg/day) for 12 weeks. The DEN-DDS-BTL group was administered betel leaf powder suspension (BTL) 200 mg/kg/day one hour before the administration of DDS for 12 weeks. At the end of the 14th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.

**Group 7: DEN-DDS (n=13)**

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<th>Week</th>
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<td>DDS 50 mg/kg (once daily)</td>
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</table>
2.2.4 Effect of BTL on DDS Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 2 groups. The animals of DEN-DDS group received once daily oral administration of DDS (50mg/kg/day) for 6 weeks. The DEN-DDS-BTL group received a suspension of BTL (200mg/kg/day) one-hour before the administration of DDS, for 6 weeks. One week after the start of the test agent and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN treatment the rats were sacrificed. A schematic representation of the treatment schedule is given below.

2.2.5 Effect of BTL on AAF Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 2 groups. The animals of DEN-AAF group received once daily oral administration of AAF (20mg/kg/day) for 6
weeks. The DEN-AAF-BTL group received suspension of BTL (200mg/kg/day) one hour before the administration of AAF, for 6 weeks. One week after the start of the test agent and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN treatment the rats were sacrificed. A schematic representation of the treatment schedule is given below.

Group 4: DEN-AAF (n=10)

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<td></td>
<td>DEN</td>
<td>PH</td>
<td>AAF 20mg/kg (once daily)</td>
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<td></td>
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<td>Sacrifice</td>
</tr>
</tbody>
</table>

Group 13: DEN-AAF-BTL (n=12)

<table>
<thead>
<tr>
<th>Week</th>
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<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<td>DEN</td>
<td>PH</td>
<td>AAF 20mg/kg + BTL 200 mg/kg (once daily)</td>
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<td>Sacrifice</td>
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</tbody>
</table>

2.2.6 Effect of BTL on PB Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The animals were administered a single i.p. injection of DEN (200mg/kg) in saline. After 2 weeks rest period, the rats were divided into 2 groups. The animals of DEN-PB group received once daily oral administration of PB (90mg/kg/day) for 6 weeks. The DEN-PB-BTL group received suspension of BTL (200mg/kg/day) one hour before the administration of PB, for 6 weeks. The DEN-BTL group received once daily oral administration of BTL (200mg/kg/day) for 6 weeks. One week after the start of the test and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.
2.2.7 Effect of BTL on the Initiation Stage of DDS Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The BTL-DEN-DDS group was administered BTL (200mg/kg/day) for five consecutive days. On the third day of BTL administration DEN (200mg/kg) was administered i.p. BTL treatment was continued for next 2 days. The DEN-DDS group was administered DEN (200mg/kg; i.p) once. After 2 weeks rest period, both the groups group received once daily oral administration of DDS (50mg/kg/day) for 6 weeks. One week after the start of the test and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.
2.2.8 Effect of BTL on the Initiation Stage of AAF Promoted Hepatocarcinogenesis in Ito Model

The study was conducted in male Charles-Foster rats. The BTL-DEN-AAF group was administered BTL (200mg/kg/day) for five consecutive days. On the third day of BTL administration DEN (200mg/kg) was administered i.p., BTL treatment was continued for next 2 days. The DEN-AAF group was administered DEN (200mg/kg; i.p) once. After 2 weeks rest period, both the groups received once daily oral administration of AAF (20mg/kg/day) for 6 weeks. One week after the start of the test and/or promoter administration, surgical partial hepatectomy was performed on all rats. At the end of the 8th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.
2.2.9 Effect of Bengal Gram on DDS Promoted Hepatocarcinogenesis in Pitot Model

The study was conducted in Female Charles Foster rats. Within 24 hours of 2/3rd partial hepatectomy surgery the rats were administered DEN (10mg/kg) p.o. After 2 weeks rest period, the rats were divided into 2 groups. Both the groups were administered DDS (50mg/kg/day) for 12 weeks. The DEN-DDS (No Gram) group was fed only the standard pellet diet, whereas the other group received standard rat pellets + Bengal gram in the diet. In the case of the DEN-DDS (No Gram) group Bengal gram was withdrawn one week before the administration of DEN and continued for duration of the experiment. At the end of the 14th week post DEN the rats were sacrificed. A schematic representation of the treatment schedule is given below.

The nutritional composition of Bengal gram

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<thead>
<tr>
<th>Nutrient</th>
<th>Content</th>
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</thead>
<tbody>
<tr>
<td>Energy</td>
<td>986 kJ</td>
</tr>
<tr>
<td>Protein</td>
<td>13 g</td>
</tr>
<tr>
<td>Fat</td>
<td>3.8 g</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>41 g</td>
</tr>
<tr>
<td>Fibre</td>
<td>17 g</td>
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**Group 7: DEN-DDS (n=13)**

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**Group 23: DEN-DDS (n=5) No Bengal gram in the diet**

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</table>
3.10 Estimation of Methaemoglobin in blood samples

Methaemoglobin in the blood was estimated fortnightly and at the time of sacrifice by the cyanide-ferricyanide method as described by Dacie and Lewis 1986. Methaemoglobin is expressed as the percent of hemoglobin that has been converted to methaemoglobin.

Reagents:
A. Phosphate buffer 0.1M, pH 6.8.
B. Detergent solution Nonidet=N 40 1%
C. Lysing buffer: 2:3 Mixture of A and B
D. Potassium cyanide solution 5% in TDW
E. Potassium ferricyanide solution 5% in TDW

Procedure:
1) The thoroughly cleaned end of the tail of rat was nipped with a sharp razor blade and blood was collected on a clean slide. 100 µl of blood was immediately transferred to a tube containing the lysing buffer kept over ice. The tube was vortexed immediately for 1 minute in order to lyse the blood cells and avoid clot formation. The lysed solution was stored over ice until separation into 2 parts.

2) The blood solution was divided into two equal parts A and B.

3) The absorbance of part A at 630nm (D1) was measured. To this 20 µl of potassium cyanide solution was added and mixed and absorbance (D2) was again measured.

4) To the part B, 20 µl of potassium ferricyanide solution was added and mixed and after 5 minutes, absorbance (D3) was measured. To this, 20 µl of potassium cyanide solution was added, mixed and absorbance measured (D4) again.

5) The measurements were made against a blank containing the lysing buffer. The time duration in between the collection of blood and measuring the absorbance of the last sample of blood was not more than 1 hour.

Calculation:
Methaemoglobin % = \{(D1 - D2)/(D3 - D4)\} * 100
exposure and protect enzyme activities. Sections 5μm in thickness were cut on a rotary microtome (A0820, USA) picked up on albumin-coated slides and dried at room temperature overnight. The sections from the acetone fixed tissues were stained immunohistochemically for Glutathione S-Transferase-P (GST-P). The sections from PBF fixed tissues were stained for iron in the hemosiderotic spleens using the Perl’s potassium ferricyanide method (Pearse, 1985). Sections of PBF fixed tissues were stained by celestine-blue-eosin for histopathological examination.

3.12 Histochemical/Immunohistchemical techniques used for staining

3.12.1 Histochemical staining for γ-glutamyl transpeptidase in cryostat sections post-fixed in cold acetone (Rutenberg, et al. 1976)

Reagents:

A. L-γ-glutamyl 4-methoxy 2-naphthylamide (0.125mg/ml)
B. Dimethyl sulphoxide
C. Sodium chloride (0.85%)
D. Tris buffer-HCl (0.25M, pH 7.4)
E. Glycylglycine (0.5mg/ml)
F. Fast Blue BB (0.5mg/ml)
G. Copper sulfate (0.1M).

Procedure:

1. Fresh cryo-sections (5μm) were fixed in ice-cold acetone for 20 minutes and air-dried.
2. The sections were ringed with silicone grease and immersed in saline.
3. Sections were incubated in the freshly prepared incubation medium containing the substrate L-γ-glutamyl 4-methoxy 2-naphthylamide (0.125mg/ml), glycylglycine (0.5mg/ml) and Fast Blue BB (0.5mg/ml) in 0.25MTris –HCl (pH 7.4) and 0.85% NaCl for 30 minutes at 37°C.
4. The incubation medium was shaken off and the slides were placed in saline to wash off the incubation medium.
3.11 Sacrifice, tissue sampling and weighing isolated organs

The rats were killed under deep ether anesthesia by decapitation. The liver, spleen and any other abnormal organ observed were immediately isolated and kept in individual containers kept on ice. The liver and spleen were immediately weighed (absolute weight). The relative organ weight of the organs isolated were calculated from the absolute weight as follows:

relative weight (g/100g body weight) = (absolute weight (g)/weight of the animal (g)) * 100

3.11.1 Tissue sampling processing and cryostat sectioning:

Representative slices of each lobe from each rat were placed on a single tissue chuck to form a composite ‘montage’ and were snap frozen in liquid nitrogen. The tissue chucks were mounted in a cryostat (Slee, England) and cryostat sections (5μm) were obtained at −25°C. Serial cryostat sections were fixed in cold (-20°C) acetone (acetone dried over sodium sulfate) for 20 minutes and acetic ethanol (1ml glacial acetic acid in 100ml absolute alcohol) for 30 minutes. The sections fixed in acetic ethanol were washed with cold absolute alcohol and the sections were air dried and stored at −20°C till staining. The acetone fixed sections were used to stain for GGT and the acetic ethanol fixed sections were stained for glycogen. Pieces of the liver were frozen and stored at −20°C and used for estimation of liver glutathione (free –SH groups) and protein.

3.11.2 Tissue sampling for histology, histochemistry and immunohistochemistry:

The liver was sliced and representative slices from each lobe were fixed in cold acetone and 4% phosphate buffered formaldehyde (PBF). Slices of the spleen were fixed in 4% PBF. The fixed tissues were dehydrated with a series of increasing concentrations (70, 90 and 100%) alcohol’s, cleared in xylene and embedded in paraffin (58-60°C congealing point). In the case of liver sections fixed in cold acetone, the tissues were transferred to 100% alcohol directly, and the time period for paraffin embedding was reduced to avoid excessive heat
exposure and protect enzyme activities. Sections 5 μm in thickness were cut on a rotary microtome (A0820, USA) picked up on albumin-coated slides and dried at room temperature overnight. The sections from the acetone fixed tissues were stained immunohistochemically for Glutathione S-Transferase-P (GST-P). The sections from PBF fixed tissues were stained for iron in the hemosiderotic spleens using the Perl’s potassium ferricyanide method (Pearse, 1985). Sections of PBF fixed tissues were stained by celestine-blue-eosin for histopathological examination.

3.12 Histochemical/Immunohistchemical techniques used for staining

3.12.1 Histochemical staining for γ-glutamyl transpeptidase in cryostat sections post-fixed in cold acetone (Rutenberg, et al. 1976)

Reagents:
A. L-γ-glutamyl 4-methoxy 2-naphthylamide (0.125mg/ml)
B. Dimethyl sulphoxide
C. Sodium chloride (0.85%)
D. Tris buffer-HCl (0.25M, pH 7.4)
E. Glycylglycine (0.5mg/ml)
F. Fast Blue BB (0.5mg/ml)
G. Copper sulfate (0.1M).

Procedure:
1. Fresh cryo-sections (5 μm) were fixed in ice-cold acetone for 20 minutes and air-dried.
2. The sections were ringed with silicone grease and immersed in saline.
3. Sections were incubated in the freshly prepared incubation medium containing the substrate L-γ-glutamyl 4-methoxy 2-naphthylamide (0.125mg/ml), glycylglycine (0.5mg/ml) and Fast Blue BB (0.5mg/ml) in 0.25M Tris –HCl (pH 7.4) and 0.85% NaCl for 30 minutes at 37° C.
4. The incubation medium was shaken off and the slides were placed in saline to wash off the incubation medium.
5. The sections were then incubated in 0.1M copper sulfate for 1 minute and thereafter rinsed in saline and then in distilled water.

6. The sections were fixed in 4% formaldehyde for 30 minutes and rinsed thoroughly in distilled water and mounted in glycerol-gelatin mountant.

GGT enzyme activity is localized as deep red azo-coupled reaction product.

3.12.2 Histochemical staining for glycogen in cryo-sections (Pearse, 1985)

Reagents:
A. Periodic acid (1%)
B. Schiff's reagent containing Pararosaniline HCl (1%)
C. Celestine blue-iron alum.

Procedure:
1. Fresh cryo-sections (5μm) were fixed in fresh 1% acetic ethanol fixative for 20 minutes and then rinsed with absolute alcohol.
2. The sections were washed in distilled water and incubated in 1-% periodic acid for 10 minutes in dark.
3. The sections were then washed in 3 changes of distilled water, each change lasting 3 minutes.
4. Sections were incubated in the Schiff’s reagent for 20 minutes in dark.
5. The sections were then washed in running water for 10 minutes and stained with celestine blue-iron alum for 1 minute.
6. The sections were then washed in running tap water for 10 minutes, dehydrated in graded ascending concentrations of alcohols (70, 90 and 100%), cleared in xylene and mounted in DPX.

Glycogen is stained deep pink by this method and nuclei are stained blue-black.

3.12.3 Immunohistochemical staining for glutathione S-transferase-p (GST-p) in acetone fixed paraffin embedded liver sections:

The method was essentially that used by Tatematsu et al. (1987). Paraffin sections of cold acetone fixed livers were used for GST-P staining.
Reagents:

A. Methanol
B. Hydrogen peroxide (0.3% in methanol)
C. Bovine serum albumin (1% and 0.3% in TDW)
D. 1st GST-P antibody (rabbit anti-human, Calbiochem)
E. 2nd antibody (goat anti-rabbit IgG)
F. Extravidin peroxidase conjugate
G. 3,3’- diaminobenzidine HCl (0.5mg/ml in Tris with 0.3% hydrogen peroxide)
H. 0.1M phosphate buffered saline (pH 7.4; PBS 7.4)
I. Saponin (0.1% in PBS),
J. Tris-HCl buffer (0.1M; pH 7.4).

Procedure:

1. The paraffin sections (5μm) were deparaffinized in xylene and rehydrated with graded series of alcohols.
2. The sections were incubated in a mixture of 3% hydrogen peroxide in methanol for 15 minutes after which they were rinsed with absolute alcohol and placed in 0.1% saponin in PBS 7.4.
3. A hydrophobic ring of silicone grease was made around the section and the sections were incubated with 0.3% bovine serum albumin (BSA) in saline for 30 minutes.
4. After shaking excess BSA, the sections were rinsed with PBS 7.4 and incubated over night at 4°C with the anti GST-P antibody (1:1000 in PBS 7.4).
5. The sections were rinsed in and transferred to PBS-saponin for 15 minutes.
6. The sections were then incubated with the biotinylated goat anti-rabbit IgG (2nd Ab; 1:20 in 1% BSA in PBS 7.4, 0.1M) for 30 minutes at room temperature.
7. The 2nd Ab was shaken off and the sections were washed in PBS-saponin solution.
8. The sections were then incubated in Extravidin-peroxidase conjugate (Extra-3, Sigma) for 30 minutes.

9. After shaking off the excess conjugate, sections were washed thrice in PBS-saponin solution (5 minutes each) and incubated in the freshly prepared chromogenic reagent containing 0.5mg/ml 3,3'-diaminobenzidine HCl in Tris-HCl buffer (pH 7.4) and 20µl hydrogen peroxide (30%) for 10 minutes.

10. The sections were rinsed thoroughly in PBS for 10 minutes, washed thrice with distilled water, dehydrated in ascending concentrations of alcohol (70, 90 and 100%), cleared in xylene and mounted in DPX. Immunoreactive GST-P is stained deep brown.

3.12.4 Staining for iron in the spleen by Perl's method (Pearse, 1985)

Sections of formaldehyde fixed spleen tissues were used for this staining.

Reagents:

A. Potassium ferrocyanide (Aqueous) 2%
B. HCl (Aqueous) 2%
C. Neutral Red (Aqueous) 1%

Procedure:

1. Sections 5µm were deparaffinized and rehydrated in descending concentrations of ethanol-distilled.

2. The sections were exposed to a fresh mixture of equal parts of 2% potassium ferrocyanide and 2%HCl for 30 minutes and washed thoroughly with distilled water.

3. The sections were counter stained with 1% aqueous neutral red solution for 10 seconds and washed with distilled water.

4. The sections were dehydrated in ascending series of alcohols, cleared in xylene and mounted in DPX.
Iron in hemosiderin deposits stained Prussian blue and the nuclei pink. The degree of hemosiderosis in spleen was assessed microscopically and graded as given below.

<table>
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<tr>
<th>No.</th>
<th>Visually observed density</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>2</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>High</td>
<td>+++</td>
</tr>
</tbody>
</table>

3.12.5 Celestine blue-Eosin staining method for general histology:

Tissue sections of formaldehyde fixed liver and spleen tissues were stained with Celestine blue-Iron alum and Eosin (CB-E) (Lillie et al. 1973) for histopathological examination.

Reagents:
A. Celestine blue (1% aqueous)
B. Iron alum (4% aqueous)
C. Eosin (1% aqueous).
D. Celestine blue and Iron Alum solutions were mixed in 1:1 proportion before use.

Procedure:
1. Sections 5μm were air dried and deparaffinized and rehydrated in a series of alcohols and water.
2. The slides were immersed in a 1:1 mixture of celestine blue and iron alum for 5 minutes.
3. The slides were washed in running tap water for 10 minutes and counter stained with 1% eosin for 1 minute.
4. The sections were washed in distilled water, dehydrated in a series of alcohols (70, 90 and 100%), cleared in xylene and mounted in DPX.

With this method, nuclei stain blue-black and the cytoplasm is stained pink.
3.13 Estimation of Glutathione (free –SH groups) in the liver

Liver glutathione (free –SH groups) was estimated essentially by the method of Ellman (1959)

Reagents:
A. Phosphate buffer (PB) 0.1M; pH 7.2
B. Tris-EDTA buffer 0.1M; pH 8.7
C. 5,5’-Dithio-bis(2-nitrobenzoic Acid) (DTNB) 0.01M
D. Standard glutathione, reduced 0.1mM

Procedure:
1. The weighed quantity of liver tissue (approx. 250mg) was taken in a homogenizing tube, 1ml phosphate buffer (PB) pH 7.2 was added to it and homogenized with 20 up and down strokes, each stroke lasting for 7 seconds. The first homogenate was poured in a pre-labeled tube, and with subsequent additions of 2, 1 and 1 ml of buffer the volume was made up to 5 ml. For each addition, the tube and the homogenizer were rinsed with PB.
2. The homogenates were centrifuged at 4000rpm for 20 minutes and the supernatant was collected.

1. 2ml Tris-EDTA buffer (pH 8.7) was taken in a tube, to this 0.9ml distilled water and 0.1ml of the supernatant was added and mixed. To this 0.1ml of 0.01M DTNB was added.
2. The mixture was incubated at 37⁰ for 20 minutes and the absorbance read at 412nm in a Shimadzu spectrophotometer.
3. Standard glutathione used at 0.1mM concentration was prepared in PB (pH 7.2).

Calculations

Estimated glutathione (mmol) = (absorbance of Unknown/absorbance of Standard) X conc. Standard.

Glutathione (free –SH groups mmol/mg protein) = {estimated glutathione/ estimated protein}
3.14 Estimation of Protein in liver tissue

The protein content in the liver tissue was estimated by the method of (Lowry et. al. 1951)

Reagents

A. Solution A: (8% Sodium carbonate)
B. Solution B: (1.2gm sodium potassium tartarate and 0.6gm copper sulfate in 1000ml DW)
C. Solution C: 1mg/ml Bovine Serum Albumin (BSA) in TDW
D. Solution D: 1.0N Folin reagent.

Procedure:

1. Solution A and solution B were mixed in 1:1 proportion. 5ml of the mixture was taken in a tube and 0.9ml TDW and 0.1ml sample (centrifuge supernatant as described in the method for glutathione estimation) were added and incubated at 37° for 10 minutes.
2. Folin reagent (0.5ml) was added to the above mix and incubated at 37° for 30 minutes. The absorbance was read at 625nm.
3. Bovine serum albumin (1mg/ml in TDW) was used as the standard protein.

Calculations

Estimated protein (mg) = (absorbance of unknown/absorbance of standard) X quantity of standard protein

The quantity of protein obtained was used for the calculation of glutathione concentration (free –SH groups mmol/mg protein) in the tissue.

3.15 Foci volume estimation

The foci/nodules selectively stained for the markers GGT and GST-P were analyzed by image analysis for the volume and number of foci. For this, the computer based image analysis software developed by Prof. H. C. Pitot and his group at the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, was used. The program utilized the stereological principles developed by Delesse (1848) for estimating the volume of foci/nodule per unit volume of liver and the method of Saltykov (1967) for estimating the number of foci/nodules per unit volume or whole liver. Both
these methods derive 3-dimensional values of structures from the two-dimensional transections. Scanned images of liver sections were first opened in Scion Image (Scion Corporation, USA) where they were calibrated, processed and the data of areas of liver sections and foci/nodules were exported into Pitot's program. After extensive calculations, on the basis of liver weights, the data of liver foci/nodules were output by the program as volume of foci/nodules as percent of liver and number of foci/nodules per liver. The sections stained for GGT, GST-P were scanned using a color flat bed scanner at 300-dpi resolution and stored to disk.

3.16 Statistical analysis:

Statistical analyses of all data were done using GraphPad Prism®. Methaemoglobin data were compared between different groups and time points using the Dunnet's multiple comparison test. To determine the effect of a treatment on the relative liver weight, relative spleen weight, liver GSH levels, foci volume and number of foci/liver the Student's t-test was applied. Differences were considered significant if p<0.05.
RESULTS
General Observations

The results of the present work are organized in such a manner that the observations and data of each study are largely self-sufficient. The data on the end point of hepatocarcinogenesis used in the present study, namely the preneoplastic foci and nodules, is derived from the image analysis of liver sections stained for specific markers (GGT and GST-p) of the lesions. Microscopic examination of these sections and other sections stained for general histology and for demonstration of glycogen stores were done to identify the general histopathological characteristics of the foci and nodules. In all the studies, foci and nodules were largely identical in their general histological and phenotype expression characteristics. These characteristics are described below but to avoid redundancy are not repeated in the description of results of each study.

Similarly hemosiderosis in the spleen was evaluated by microscopic examination of the sections. The evaluation was semi-quantitative with the abundance of hemosiderin-loaded histiocytes graded as 'none' (-), low (+), moderate (++) and high (+++). The microscopic appearance of dose dependent splenic siderosis of different grades is shown in Figs. 3, 4, 5 and 6.

Preneoplastic foci/nodules observed in liver sections:

The terms preneoplastic ‘foci’ and ‘nodules’ were used in the present study to describe lesions that showed certain characteristics. Foci were small lesions and less than one liver lobule in size whereas
nODULES OCCUPY AN AREA EQUIVALENT IN SIZE TO THAT OF MORE THAN ONE LOBULE.

CLEAR CELL FOCUS:

These foci/nodules contain glycogen-storing cells that look largely 'empty' when conventionally processed and stained. Very few foci or nodules containing only clear cells were found in the present study. Mostly mixed cell foci/nodules containing clear cells and other types of cells were found. A mixed clear cell focus/nodule is shown in Figs. 7 and 8. The presence of rich glycogen stores in the cells could be seen when acetic-ethanol fixed cryostat sections are stained with PAS (Figs. 9 and 10).

THE ACIDOPHILIC CELL-FOCI:

These foci showed cells with ground glass appearance and varying degrees of eosinophilia, a characteristic of this type of foci/nodules. These foci/nodules were observed in the DEN-AAF, DEN-PB and DEN-DDS groups. An acidophilic cell nodule and mixed cell nodule showing both acidophilic and some basophilic cells are shown in Figs. 11 and 12.

BASOPHILIC CELL FOCI:

Foci/nodules with cells showing varying degrees of increased basophilia were also found DEN-AAF, DEN-PB and DEN-DDS groups (Figs. 7, 13, 14 and 15). The cells showing vesicular nuclei with prominent nucleolus were mostly observed in the DEN-AAF group but a few were also seen in the DEN-PB and DEN-DDS groups. The diffusely basophilic foci poor in glycogen represent a late type of FAH, resulting from sequential cellular changes, which lead
from clear and acidophilic foci through different stages to the basophilic cell population that prevails in hepatocellular carcinomas.

Mixed cell foci/nodules:

Foci/nodules containing more than one cell type were recognized as mixed cell foci/nodules. These were the predominant type found in the present study. A mixed clear cell and acidophilic cell nodule is shown in Figs. 7 and 8 and another with basophilic cells and acidophilic cells is shown in Figs. 11 and 14.

Other lesions:

In addition to above certain lesions known to be induced by AAF in DEN-initiated livers were also observed in DEN-AAF group. Some of these lesions are: nuclear atypia in nodules and 'oval' cell proliferation, (Fig. 17) pseudoductal formations and cystic changes (Fig. 16). In a few cases the nodules also showed evidences of necrosis, apoptosis and early spongiotic/steatotic changes (Fig. 18).

The gamma glutamyl transpeptidase (GGT) expressing foci/nodules:

Almost all the foci/nodules of the histologic types described above were also rich in GGT and so GGT-positive lesions were used in the study to quantitate foci/nodules. GGT-positive foci/nodules were easily detectable in all the groups of the study. PB caused an increased expression of this enzyme even in the portal tracts, but staining in the foci is more intense, hence the differentiation was easy. Typical GGT expressing foci/nodules are shown in Plate I and Figs. 19 and 20.
Plate I Low power overview of composite cryosection of 4 liver slices from a rat of DEN-PB (Ito model) group stained for gamma glutamyl tranferase (GGT) activity. Discrete GGT-positive foci/nodules (deep red) can be readily recognized.
Plate. II Low power overview of paraffin section of a liver slice from a rat of DEN-PB (Ito model) group immunostained for glutathione S-transferase-placental form (GST-P). Discrete GST-P positive foci/nodules (deep brown) can be readily recognized.
Fig. 3  Spleen from DEN-Vehicle Group showing normal lymphoid follicles (arrow heads) and red pulp (arrow). No hemosiderin was seen in the histiocytes (*Hemosiderosis Grade: 0/none*); nuclei are stained red. Perl's iron-neutral red, X63

Fig. 4  Red pulp area of spleen from DEN-DDS10 Group. A few histiocytes show hemosiderin (Prussian blue) deposits (*Hemosiderosis Grade: +/low*); nuclei are stained red. Perl's iron-neutral red, X160.
Fig. 5 Red pulp area of spleen from DEN-DDS25 Group. Several histiocytes show hemosiderin (Prussian blue) deposits (Hemosiderosis Grade: ++/moderate); nuclei are stained red. Perl's iron-neutral red, X250

Fig. 6 Red pulp area of spleen from DEN-DDS50 Group. Almost all the histiocytes show hemosiderin (Prussian blue to blue-black) deposits (Hemosiderosis Grade: +++/high); nuclei are stained red. Perl's iron-neutral red, X250
Fig. 7  A *mixed cell* nodule (arrow) with 'clear' and basophilic cells and a *basophilic* cell nodule (yellow arrowhead) in the liver of a DEN-AAF (Ito's model) group rat. Celestine blue-eosin. X63.

Fig. 8  Higher magnification of 'boxed' area in Fig. 7. The nodule has 'clear' cells (yellow arrows), basophilic cells and some cells undergoing steatotic/ early 'spongiotic' changes (arrow). Celestine blue-eosin, X250.
Fig. 9  Mixed cell foci and nodules containing several glycogen-storing cells (deep pink; arrows) in the liver of a DEN-AAF (Ito's model) group rat. Acetic-ethanol post-fixed fresh cryosection, PAS-H, X63.

Fig. 10  A mixed cell focus/nodules containing predominantly glycogen-storing cells (deep pink; arrow) in the liver of a DEN-AAF (Ito's model) group rat. Acetic-ethanol post-fixed fresh cryosection, PAS-H, X250
**Fig. 11** A mixed cell nodule of basophilic and acidophilic cells (arrow) and an acidophilic nodule (white arrow) in the liver of a DEN-AAF group (Ito's model) rat. Celestine blue-eosin. X63.

**Fig. 12** A large mixed cell nodule of acidophilic and some basophilic cells (white arrow) in the liver of a DEN-AAF group (Ito's model) rat. Celestine blue-eosin, X63.
Fig. 13 Part of a *mixed cell* nodule showing acidophilic (yellow arrow), basophilic (white arrowhead) and a few 'clear' (white arrow) cells. Celestine blue-eosin, X250.

Fig. 14 Part of the mixed cell nodule showing both acidophilic (arrows) and basophilic (white arrowheads) cells. Celestine blue-eosin. X250.
Fig. 15 A *basophilic* cell nodule (arrow) showing varying degrees of basophilia. Celestine blue-eosin, X250.

Fig. 16 Pseudoductal formation (black arrows) in and around a nodule (yellow star) and cystic changes (white arrows) in a DEN-AAF group rat. Celestine blue-eosin, X250.
Fig. 17 A nodule showing nuclear atypia. Considerable 'oval' cell proliferation (arrow) is also seen. Liver from a DEN-AAF group rat. Celestine blue-eosin, X250.

Fig. 18 A nodule showing cells undergoing apoptosis (arrowheads) and early spongiotic/steatotic changes (arrow) in the liver of a DEN-AAF (Ito's model) group rat. Celestine blue-eosin, X250.
Fig. 19 GGT-positive foci and nodules (arrows). The surrounding normal liver parenchyma is GGT-negative except the bile canaliculi. Fresh cryosections stained for GGT, not counterstained. X63

Fig. 20 Two GGT-positive foci/nodules (arrows). Bile ductules (arrowheads) are also characteristically positive. Fresh cryosections stained for GGT, not counterstained. X160
Fig. 21 Liver section showing foci/nodules (arrows) expressing glutathione-S-transferase (placental form; GST-P). Surrounding normal liver parenchyma is devoid of the enzyme protein. DEN-AAF group rat. Immunohistochemical staining; not counterstained. X63.

Fig. 22 GST-P expressing cells (deep brown) in a nodule at higher magnification. Surrounding normal liver parenchyma is devoid of the enzyme protein. DEN-AAF group rat. Immunohistochemical staining; not counterstained. X160.
Glutathione S-Transferase-P expressing foci:

Almost all the foci/nodules of different histologic types were positive for GST-P also. The normal surrounding liver parenchyma was devoid of GST-p. Liver foci/nodules positive for GST-P are shown in Plate II and Figs. 21 and 22.

4.1. PROMOTION OF DEN-INITIATED HEPATOCARCINOGENESIS BY DAPSONE (DDS)

4.1.1. Comparative Study on the Promotion Efficacy of Dapsone (DDS) and the Standard Promoters 2-Acetamidoflourene (AAF) and Phenobarbitone (PB) in the Ito Model of Hepatocarcinogenesis

4.1.1.1. General health conditions (Cage side observations and body weight)

The DEN-DDS group showed a better health status as compared to the DEN-AAF and DEN-PB treated groups. The DEN-AAF rats had unkempt fur and were sluggish. No detectable abnormality was observed in the DEN-VEHICLE, DEN-DDS and DEN-PB groups.

A steady gain in body weight was seen in the DEN-VEHICLE, DEN-DDS and DEN-PB groups (Fig. 23). The DEN-DDS treated rats showed a better gain in body weight than the vehicle treated rats, especially from week 2 post DEN till the end of the observation period (week 8). The rats of DEN-AAF group did not gain weight throughout the experiment.

4.1.1.2. Methemoglobin

A clear increase in methemoglobin concentration was seen in the DEN-DDS group starting from week 2 post DDS administration (Fig.
The values increased steadily till the 4th week of DDS administration, but decreased by the end of the observation period (week 8). However, the values were still significantly higher compared to the vehicle group.

4.1.4.3. Liver weights

The relative liver weight in DEN-DDS group was not significantly different from that of DEN-VEHICLE group. But there was a significant increase in the relative liver weights in the DEN-PB and DEN-AAF (p<0.05; Fig. 25) groups.

4.1.4.4. Spleen weights

The DEN-DDS group had a significant increase (p<0.001) in the relative spleen weights, whereas, DEN-PB and DEN-AAF groups had spleen weights almost similar to that of the vehicle group (Fig. 26).

The spleen in the DEN-DDS group was siderotic with the red pulp showing accumulations of hemosiderin in the histiocytes. No hemosiderosis was observed in the DEN-VEHICLE, DEN-PB and DEN-AAF groups. The degree of hemosiderosis visually assessed and graded on a semi-quantitative scale is shown in the table below.

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<th>No.</th>
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<tr>
<td>1</td>
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<td>-</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS50</td>
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4.1.5. Liver Glutathione levels

There was slight decrease in the total liver GSH content in the DEN-PB group, though this decrease was not statistically significant (Fig.
The DEN-AAF group showed an insignificant increase while the DEN-DDS treated group had almost similar levels of glutathione to that of DEN-VEHICLE group.

4.1.1.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 28, 29, 30 and 31.

4.1.1.6.1. Volume of foci/nodules

The volume of DEN initiated preneoplastic foci/nodules expressed as % of liver was significantly increased in the DEN-DDS, DEN-PB (p<0.05) and AAF (p<0.01). The volume of foci in the DEN-DDS group was 103%, DEN-PB 680% and DEN-AAF group was 986% more than that in the DEN-VEHICLE group. (Fig. 32).

4.1.1.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS group was significantly higher than in the DEN-VEHICLE group (p<0.05). PB and AAF treatment did not show any increase in the number of foci/liver (Fig. 33).
Fig. 23  Showing gain/loss in body weight in DEN-initiated and DDS (DEN-DDS), PB (DEN-PB) and AAF (DEN-AAF)-promoted and VEHICLE (DEN-VEHICLE) treated control animals. Model: Ito's - Administration of promoter begins at week 2 - Partial hepatectomy.

Fig. 24  Showing weekly methemoglobin levels of DEN-initiated and DDS-promoted (DEN-DDS) and VEHICLE (DEN-VEHICLE) treated control animals. Model: Ito's 
* = p<0.05,  ** = p<0.01,  *** = p<0.001
Fig. 25  Showing relative liver weights of DEN-initiated and DDS (DEN-DDS), PB (DEN-PB), AAF (DEN-AAF) promoted, and VEHICLE (DEN-VEH) treated animals.
Model: Ito s.
* =p<0.05

Fig. 26  Showing relative spleen weights of DEN-initiated and DDS (DEN-DDS), PB (DEN-PB), AAF (DEN-AAF) promoted, and VEHICLE (DEN-VEH) treated animals.
Model: Ito s.
*** =p<0.001
Fig. 27  Showing liver GSH levels of DEN-initiated (DEN-VEH) and DDS, PB and AAF-promoted (DEN-DDS), (DEN-PB) and (DEN-AAF) animals. Model: Ito s.
Fig. 28  Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-VEHICLE group (Ito's model).

Fig. 29  Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS50 group (Ito's model).
**Fig. 30** Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-PB group (Ito’s model).

**Fig. 31** Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-AAF group (Ito’s model).
Fig. 32 Showing liver foci volume in DEN-initiated and DDS (DEN-DDS), PB (DEN-PB) and AAF (DEN-AAF)-promoted and vehicle control (DEN-VEH) animals. Model: Ito’s.

* = p<0.05, ** = p<0.01

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Fig. 33 Showing number of foci/liver in DEN-initiated and DDS (DEN-DDS), PB (DEN-PB) and AAF (DEN-AAF)-promoted and vehicle control (DEN-VEH) animals. Model: Ito’s.

* = p<0.05
4.1.2. Dose Response Study of DDS in Pitot Model of Hepatocarcinogenesis

4.1.2.1. General health conditions (Cage side observations, body weight and food consumption)

Except for one rat from the DDS 10mg/kg group and one from the 50mg/kg group, which were sluggish and showed unkempt fur, there were no abnormalities in the external appearance and behavior of the rats in any of the groups.

A steady gain in body weight was seen in all the groups (Fig. 34). Although not significant, all the DDS 25mg/kg group showed a lesser gain in body weight than the vehicle treated group, especially from week 2 post DEN and partial hepatectomy till the end of the observation period (week 12).

This difference in body weight between the groups appeared to be broadly related to the food consumption in these groups, (Fig. 35) where all the DDS treated rats showed relatively lesser food consumption than DEN-VEHICLE group.

4.1.2.2. Methemoglobin

A clear dose related increase in methemoglobin concentration was seen in the DDS treated groups starting from week 2 post DDS administration (Fig. 36). However the values decreased more or less steadily by week 12 of DDS administration when only the values in the DDS 25mg/kg and DDS50 mg/kg remained significantly higher than the vehicle treated control group.
4.1.2.3. Liver weight

The relative liver weight in the DDS treated groups was not significantly different from that found in the vehicle treated group (Fig. 37) and no dose-related changes in the liver weight was observed.

4.1.2.4. Spleen weight

The DDS 50mg/kg group had a slight decrease in the spleen weights while in the other groups there was no significant difference as compared to the DEN-VEHICLE group (Fig. 38).

The degree of splenic hemosiderosis in the four groups is shown in the table below. A dose related increase in the hemosiderosis was observed with the DEN-DDS50 group showing the maximum siderosis followed by DEN-DDS25 and DEN-DDS10. No siderosis was detectable in the DEN-VEHICLE group.

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<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
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<td>+++</td>
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4.1.2.5. Liver Glutathione (GSH) levels

There was dose-related decrease in the total liver GSH content in the DDS treated groups (Fig. 39). However, this decrease was statistically significant only in the DDS 50mg/kg group but not in the
DDS 10mg/kg and DDS 25mg/kg dose groups as compared with the vehicle group.

4.1.2.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot’s program during analysis is shown in Figs. 40, 41, 42 and 43.

4.1.2.6.1. Volume of foci/nodules

DDS increased the volume of preneoplastic foci/nodules in a dose-related manner (Fig. 44). The DEN-DDS 10mg/kg group had 96.5%, DEN-DDS 25mg/kg group had 355.5% and the DEN-DDS 50mg/kg had 158.13% more foci volume than DEN-VEHICLE treated group. However there was no significant difference in the volume of foci between DDS 25mg/kg and DDS 50mg/kg dose groups.

4.1.2.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver found in the DEN-DDS 10mg/kg, 25mg/kg and 50mg/kg were not significantly different from that of the DEN-vehicle group (Fig. 45).
Fig. 34  Showing gain/loss in body weight in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot's Administration of promoter

Fig. 35  Showing weekly average food consumption per rat in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot's Administration of promoter
Fig. 36  Showing weekly methemoglobin levels in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot’s.

* =p<0.05, ** =p<0.01, *** =p<0.001

Fig. 37  Showing relative liver weights in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot’s
Fig. 38 Showing relative spleen weights in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Piotot's
* = p<0.05

Fig. 39 Showing liver glutathione levels in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Piotot's
* = p<0.05
Overlay of Tissue Images with 4 Markers.
Exp ID: DEN-Agar-PitotCFF, Image+MSVB6, 12-08-2002, CNJ-PSRM
File ID: 806
Rat: ct806
Liver wt.: 8.467

Marker Color:
- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.7788
3: 0.0000
4: 0.0000

Fig. 40 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-VEHICLE (Agar) group (Pitot's model).

Overlay of Tissue Images with 4 Markers.
Exp ID: DEN-DDS10PitotCFF, Image+MSVB6, 12-08-2002, CNJ-PSRM
File ID: 760
Rat: ct760
Liver wt.: 4.605

Marker Color:
- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 1.3745
3: 0.0000
4: 0.0000

Fig. 41 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS10 group (Pitot's model)
Overlay of Tissue Images with 4 Markers.
File ID: 759
Rat: cr759
Liver wt.: 6.346

Marker Color:
- 1: S
- 2: R, GGT
- 3: Y
- 4: G

Tis. Area, sq cm
1: 0.0000
2: 2.2603
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 42 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS25 group (Pitot's model).

Overlay of Tissue Images with 4 Markers.
Exp ID: DEN-DDS50-PitotCFF: Image+MSVB6; 12-08-2002; CNJ-PSRM
File ID: 767
Rat: cr767
Liver wt.: 6.573

Marker Color:
- 1: S
- 2: R, GGT
- 3: Y
- 4: G

Tis. Area, sq cm
1: 0.0000
2: 2.4473
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 43 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS50 group (Pitot's model).
Fig. 44 Showing liver foci volume in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot's

* = p<0.05

Fig. 45 Showing number of foci/liver in DEN-initiated DDS promoted at various doses 10mg/kg (DEN-DDS10), 25mg/kg (DEN-DDS25), 50mg/kg (DEN-DDS50) and VEHICLE (DEN-VEHICLE) treated animals Model: Pitot's
4.2. MODULATION OF DDS PROMOTED HEPATOCARCINOGENESIS

4.2.1. Modulation by Cytochrome P450 Isoenzyme Inhibitors

4.2.1.1. Cimetidine (CMT; a CYP 3A4 inhibitor) and Tolbutamide (TLB; a CYP 2C9 inhibitor) on DDS Promoted Hepatocarcinogenesis in Pitot Model

4.2.1.1.1. General health conditions (Cage side observations, body weight and food consumption)

Rats in all the groups were healthy throughout the observation period except for one rat of the DEN-DDS group that showed unkempt fur and was sluggish from the 2nd week of DDS administration till the end of the observation period.

A steady gain in body weight was seen in all the groups (Fig. 46). However, the DEN-DDS-TLB group showed a lesser gain in body weight than the DEN-DDS and DEN-DDS-CMT groups, especially from week 2 post DEN and partial hepatectomy till the end of the observation period (week 12).

The difference in body weight between the groups appears to be broadly related to the food consumption. The DEN-DDS-TLB group showed relatively lesser food consumption than the DEN-DDS group (Fig. 47).

4.2.1.1.2. Methemoglobin

The methemoglobin concentration showed a transient increase in all the groups compared to the DEN-VEHICLE group. There were no significant differences in methemoglobin concentration seen in the DEN-DDS, DEN-DDS+TLB and DEN-DDS+CMT groups starting from week 2 post DDS administration (Fig. 48). The values increased
steadily till the 4th week of DDS administration, but gradually
decreased by the end of the observation period (week 12). Concurrent
administration of CMT along with DDS (DEN-DDS-CMT) induced
a reduction in the methemoglobin levels by week 12, though the
decrease was not significant.

4.2.1.1.3. Liver weights

No significant difference was observed in the relative liver weights of
DEN-DDS, DEN-DDS-TLB and DEN-DDS-CMT groups (Fig. 49).

4.2.1.1.4. Spleen weights

DEN-DDS-TLB group showed a significant increase (p<0.05) in the
relative spleen weight than the DEN-DDS treated control rats. There
was no significant difference in the relative spleen weights of the
DEN-DDS and DEN-DDS-CMT groups (Fig. 50).

As shown in the table below, hemosiderin deposition was less in the
DEN-DDS-CMT and DEN-DDS-TLB groups as compared to the
DEN-DDS group. DEN-DDS-CMT had the least deposition of
hemosiderin followed by DEN-DDS-TLB.

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS-TLB</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>DEN-DDS-CMT</td>
<td>+</td>
</tr>
</tbody>
</table>

4.2.1.1.5. Liver Glutathione levels

A slight decrease in the total liver GSH content in the DEN-DDS-
TLB and DEN-DDS-CMT groups as compared with the DEN-DDS.
group was observed. The decrease was however not significant (Fig. 51).

4.2.1.1.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 52, 53 and 54.

4.2.1.1.6.1. Volume of foci nodules

Both the DEN-DDS-TLB and DEN-DDS-CMT groups showed significantly reduced volume of foci than the DEN-DDS group (p<0.01). The reduction in the foci volume in both the DEN-DDS-TLB and DEN-DDS-CMT groups was almost similar (Fig. 55). The DEN-DDS-TLB group had 68% and DEN-DDS-CMT 72% reduced foci volume than the DEN-DDS group.

4.2.1.1.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS-TLB and DEN-DDS-CMT groups were significantly less than the DEN-DDS group (p<0.05) (Fig. 56). The reduction in the number of foci/liver in both the DEN-DDS-TLB and DEN-DDS-CMT groups was not significantly different.
Fig. 46 Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Pitot's. Administration of promoter (and modulator) begins at week 2.

Fig. 47 Showing weekly food consumption in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Pitot's. Administration of promoter (and modulator) begins at week 2.
Fig. 48  Showing weekly changes in methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS and VEHICLE (DEN-VEHICLE) treated animals. Model: Pitot's.

Fig. 49  Showing relative liver weights of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS Model Pitot's.
Fig. 50  Showing relative spleen weights of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model Pitot's.

* = p<0.05

Fig. 51  Showing liver glutathione levels of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model Pitot's
Fig. 52  Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS50 group (Pitot's model).

Fig. 53  Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-TLB group (Pitot's model).
Overlay of Tissue Images with 4 Markers.

Exp ID: DEN-DDS50-CMT50-PitoCFF. Image+MSVB6; 12-14-2002; CNJ-PSRM
File ID: 708
Rat: CR708
Liver wt.: 7.601

Marker Color:
- 1: S
- 2: R, GGT
- 3: Y
- 4: G

Tissue Area: sq cm
1: 0.0000
2: 2.5309
3: 0.0000
4: 0.0000

Fig. 54 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-CMT group (Pitot's model).
Fig. 55  Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Pitot's ** = p<0.01

Fig. 56  Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Pitot's *= p<0.05
4.2.1.2. Cimetidine (CMT; a CYP 3A4 inhibitor) and Tolbutamide (TLB; a CYP 2C9 inhibitor) on DDS Promoted Hepatocarcinogenesis in Ito Model

4.2.1.2.1. General health conditions (Cage side observations and body weight)

All the rats were active and healthy throughout the observation period and no detectable abnormality was observed in their behavior during the period of the experiment.

A steady gain in body weight was seen in all the groups (Fig. 57). The DEN-DDS-TLB group showed a lesser gain in body weight than the DEN-DDS and DEN-DDS-CMT treated rats, especially from week 2 post DEN and partial hepatectomy till the end of the observation period (week 8).

4.2.1.2.2. Methemoglobin

There was a steady increase in the methemoglobin levels in the DEN-DDS and the DEN-DDS-TLB treated groups till the week 4. In contrast the DEN-DDS-CMT group showed a significantly reduced (p<0.001) methemoglobin levels as compared to the DEN-DDS control group throughout the observation period. By the 6th week of DDS treatment the reduction in the methemoglobin levels was observed in the DEN-DDS-TLB but was not significantly different from that shown by the DEN-DDS group (Fig. 58).

4.2.1.2.3. Liver weights

No significant differences were found between the relative liver weights of the DEN-DDS, DEN-DDS-TLB and DEN-DDS-CMT groups (Fig. 59).
4.2.1.2.4. Spleen weights

There was a decrease in the relative spleen weight of the DEN-DDS-CMT group rats, but this was not statistically significant when compared to the DEN-DDS group (Fig. 60).

As shown in the table below, hemosiderin deposition was less in the DEN-DDS-CMT group as compared to the DEN-DDS group. DEN-DDS-TLB group had deposition of hemosiderin comparable to the DEN-DDS group.

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS-TLB</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>DEN-DDS-CMT</td>
<td>+</td>
</tr>
</tbody>
</table>

4.2.1.2.5. Liver Glutathione levels

A slight decrease in the total liver GSH content in the DEN-DDS-TLB group was observed which was however not significant. The DEN-DDS-CMT group showed a significant decrease in the glutathione content (p<0.05) compared to the DEN-DDS group (Fig. 61).

4.2.1.2.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot’s program during analysis is shown in Figs. 62, 63 and 64.

4.2.1.2.6.1. Volume of foci/nodules

Both the DEN-DDS-TLB and DEN-DDS-CMT groups showed a significantly reduced volume of foci compared to the DEN-DDS
group \( (p<0.01, p<0.05 \text{ respectively}) \). The DEN-DDS-TLB group had
68.24\% reduced foci volume and the DEN-DDS-CMT group had
71.95\% reduced foci volume than the DEN-DDS group (Fig. 65).

4.2.1.2.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS-TLB and
DEN-DDS-CMT groups were significantly less than that found in the
DEN-DDS group (Fig. 66). DEN-DDS-TLB group showed a greater
reduction in the number of foci/liver than the DEN-DDS-CMT
group.
Fig. 57  Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito’s. ▲ Administration of promoter (and modulator) begins at week 2, ▼ Partial hepatectomy

Fig. 58  Showing weekly methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito’s. *** = p<0.001
Fig. 59  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito’s.

Fig. 60  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito’s.
Fig. 61  Showing liver glutathione levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito's  
** = p<0.01
Overlay of Tissue Images with 4 Markers
Exp ID: DEN-DDS50-ItoCFM; Image+MSVB6; 12-15-2002; CNJ-PSRM
File ID: 6671
Rat: CR667
Liver wt.: 10.49g

Marker Color:

- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 3.1736
3: 0.0000
4: 0.0000

Fig. 62 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Ito's model).

Overlay of Tissue Images with 4 Markers
Exp ID: Gr2DEN-DDSB0-TLB50-ItoCFM; Image+MSVB6; 11-26-2002; CNJ-PSRM
File ID: 675
Rat: CR675
Liver wt.: 12.43g

Marker Color:

- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 3.2589
3: 0.0000
4: 0.0000

Fig. 63 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-TLB group (Ito's model)
Fig. 64 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-CMT group (Ito's model).
Fig. 65 Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito's. * = p<0.05, ** = p<0.01

Fig. 66 Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with tolbutamide (DEN-DDS-TLB) and cimetidine (DEN-DDS-CMT) simultaneously with DDS. Model: Ito's. * = p<0.05, ** = p<0.01
4.2.1.3. Effect of TLB (a CYP 2C9 Inhibitor) on PB Promoted Hepatocarcinogenesis in Ito Model

4.2.1.3.1. General health conditions (Cage side observations and body weight)

Rats in all the groups were healthy throughout the observation period, except for one rat of the DEN-PB-TLB group, which showed unkempt fur and was sluggish. The rats belonging to the DEN-PB group were normal.

A steady gain in body weight was seen in all the groups (Fig. 67). The DEN-PB-TLB treated rats showed a lesser gain in body weight than the DEN-PB group. There was a decrease in the gain after partial hepatectomy in the DEN-PB-TLB group, but one week after partial hepatectomy the rats gained weight.

4.2.1.3.2. Liver weights

A significant decrease in the relative liver weights was observed in the DEN-PB-TLB group as compared to the DEN-PB group (p<0.05) (Fig. 68).

4.2.1.3.3. Spleen weights

No significant difference was observed in the relative spleen weights between DEN-PB and DEN-PB-TLB groups of rats (Fig. 69).

4.2.1.3.4. Liver Glutathione levels

A slight increase in the total liver GSH content was observed in the DEN-PB-TLB group but this was not statistically significant when compared with the DEN-PB group (Fig. 70).
4.2.1.3.5. Liver preneoplastic foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 71 and 72.

4.2.1.3.5.1. Volume of foci/nodules

The DEN-PB-TLB group showed a significantly reduced (p<0.001, 90%) volume of foci than the DEN-PB group (Fig. 73).

4.2.1.3.5.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-PB-TLB group was significantly less (p<0.05) than that found in the DEN-PB group (Fig. 74).
Fig. 67 Showing gain/loss in body weight in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model: Ito's. Administration of promoter (and modulator) begins at week 2. Partial hepatectomy. Model Ito's.

Fig. 68 Showing relative liver weights in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model: Ito's. * = p<0.05
Fig. 69  Showing relative spleen weights in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model Ito's.

Fig. 70  Showing liver glutathione levels in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model: Ito's
Fig. 71 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-PB group (Ito's model).

Fig. 72 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-PB-TLB group (Ito's model).
Fig. 73  Showing liver foci volume in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model: Ito’s *** = p<0.001

Fig. 74  Showing number of foci/liver in DEN-initiated and PB-promoted (DEN-PB) animals treated with tolbutamide (DEN-PB-TLB) simultaneously with PB. Model: Ito’s * = p<0.05
4.2.2. MODULATION BY NATURAL PRODUCTS

4.2.2.1. Effect of Picroliv (PLV) on DDS Promoted Hepatocarcinogenesis in Pitot Model

4.2.2.1.1. General health conditions (Cage side observations, body weight and food consumption)

All the rats of DEN-DDS and DEN-DDS-PLV groups were active and healthy, except for one rat belonging to the DEN-DDS group, which had an unkempt fur and was inactive throughout the observation period.

A steady gain in body weight was seen in both the groups (Fig. 75).

The DEN-DDS group showed lesser food consumption between week 6 and 8 post DEN, after which it increased and was better than that shown by DEN-DDS-PLV group from wk 11 onwards (Fig. 76).

4.2.2.1.2. Methemoglobin

There was a steady rise in the methemoglobin levels in DEN-DDS and DEN-DDS-PLV groups till the week 4. The DEN-DDS-PLV group showed a significantly reduced methemoglobin level in the 2nd and 6th week (p<0.05) as compared to the DEN-DDS group. By the 8th week, methemoglobin levels increased in both the groups but were reduced by the 12th week of DDS administration. There was no significant difference in the methemoglobin levels observed in the DEN-DDS and DEN-DDS-PLV groups during the 8-12 week period (Fig. 77).

4.2.2.1.3. Liver weights

No significant differences in the relative liver weights were observed between DEN-DDS and DEN-DDS-PLV groups (Fig. 78).
4.2.2.1.4. Spleen weights

There was a slight but significant (p<0.05) increase in the relative spleen weights in the DEN-DDS-PLV group compared to the DEN-DDS group (Fig. 79).

The DEN-DDS-PLV group showed a reduced degree of hemosiderosis than the DEN-DDS group, as shown in table

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</tr>
<tr>
<td>2</td>
<td>DEN-DDS-PLV</td>
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</table>

4.2.2.1.5. Liver Glutathione levels

A highly significant (p<0.001) decrease in the total liver GSH content was observed in DEN-DDS-PLV group (Fig. 80).

4.2.2.1.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 81 and 82.

4.2.2.1.6.1. Volume of foci/nodules

The DEN-DDS-PLV group showed a significantly reduced (p<0.05) focal volume compared to DEN-DDS group (Fig. 83). The reduction was 66.67% of the foci volume of DEN-DDS group.

4.2.2.1.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS-PLV group was slightly less than the DEN-DDS group, (Fig. 84) but the difference was not statistically significant.
Fig. 75  Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's Administration of promoter (and modulator) begins at week 2.

Fig. 76  Showing average daily food consumption per rat in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's Administration of promoter (and modulator) begins at week 2.
Fig. 77 Showing weekly methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot’s.
* = p<0.05, ** = p<0.01

Fig. 78 Showing relative liver weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot’s
Fig. 79  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's
* = p<0.05

Fig. 80  Showing liver glutathione levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's
*** = p<0.001
Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-PitotCFF; Image+MSVB6: 12-08-2002; CNJ-PSRM
File ID: 757
Rat: cr757
Liver wt.: 6.573

Marker Color:
--- 1: S,
--- 2: R. GGT
--- 3: Y,
--- 4: G,

Tis. Area; sq cm
1: 0.0000
2: 2.4473
3: 0.0000
4: 0.0000

Fig. 81 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Pitot's model).

Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-PLV10-PitotCFF; Image+MSVB6: 12-13-2002; CNJ-PSRM
File ID: 730
Rat: CR730
Liver wt.: 8.958

Marker Color:
--- 1: S,
--- 2: R. GGT
--- 3: Y,
--- 4: G,

Tis. Area; sq cm
1: 0.0000
2: 2.1652
3: 0.0000
4: 0.0000

Fig. 82 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-PLV group (Ito's model).
Fig. 83 Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's
* = p<0.05

Fig. 84 Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Pitot's
4.2.2.2. Effect of PLV on DDS Promoted Hepatocarcinogenesis in Ito Model

4.2.2.2.1. General health conditions (Cage side observations, body weight and food consumption)

All the rats in both DEN-DDS and DEN-DDS-PLV groups were active and healthy throughout the observation period. Both the groups showed a steady gain in body weight during the 8-weeks observation period (Fig. 85). However the gain was much less in the DEN-DDS-PLV group from week 3 onwards in comparison to the DEN-DDS group.

4.2.2.2.2. Methemoglobin

PLV administration along with DDS completely inhibited (p<0.01, 0.001 and 0.05 at wk 2, 4 and 8, respectively) any rise in the methemoglobin concentration during the observation period (Fig. 86).

4.2.2.2.3. Liver weights

The DEN-DDS-PLV group had a significantly lower (p<0.01) relative liver weight than the DEN-DDS group (Fig. 87).

4.2.2.2.4. Spleen weights

The DEN-DDS-PLV group showed a significantly reduced (p<0.01) relative spleen weight compared to DEN-DDS group (Fig. 88).

As shown in the table below, hemosiderin deposition was less in the DEN-DDS-PLV group as compared to the DEN-DDS group.
<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS-PLV</td>
<td>+</td>
</tr>
</tbody>
</table>

4.2.2.2.5. *Liver Glutathione levels*

There was a marginal increase in the total liver GSH level in the DEN-DDS-PLV group, but the increase was not statistically significant (Fig. 89).

4.2.2.2.6. *Liver preneoplastic foci/nodules*

A representative plot picture of the composite montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 90 and 91.

3:2.2.2.6.1. *Volume of foci/nodules*

The DEN-DDS-PLV group showed a significantly reduced (p<0.05) focal volume compared to DEN-DDS group (Fig. 92). The DEN-DDS-PLV group had 51% less foci volume than the DEN-DDS group.

3:2.2.2.6.2. *Numerical density of foci/nodules in liver*

The total number of foci/liver in the DEN-DDS-PLV group was less than the DEN-DDS group (Fig. 93); however the decrease was not statistically significant.
Fig. 85  Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito's. Administration of promoter (and modulator) begins at week 2. ▲ Partial hepatectomy

Fig. 86  Showing weekly methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model Ito’s. * = p<0.05, ** = p<0.01, *** = p<0.001
Fig. 87  Showing relative liver weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito’s .

** = p<0.01

Fig. 88  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito’s

** = p<0.01
Fig. 89 Showing liver glutathione levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito’s
Fig. 90 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Ito's model).

Fig. 91 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-PLV group (Ito's model).
Fig. 92  Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito’s.

* = p<0.05

Fig. 93  Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with picroliv (DEN-DDS-PLV) simultaneously with DDS. Model: Ito’s.
4.2.2.3. Effect of Betel Leaf (BTL) on DDS Promoted Hepatocarcinogenesis in Pitot Model

4.2.2.3.1. General health conditions (Cage side observations, body weight and food consumption)

With the exception of one rat in DEN-DDS-BTL group that showed unkempt fur and one rat of DEN-DDS group that was sluggish and inactive throughout the observation period, all the rats of both DEN-DDS and DEN-DDS-BTL groups were active and healthy throughout the observation period.

A steady gain in body weight was seen in both the groups (Fig. 94). The DEN-DDS-BTL group showed a slight loss in body weight after beginning BTL administration, but later improved, and by the end of the observation period (week 12) was almost similar to the DEN-DDS group.

The DEN-DDS-BTL group showed reduced food consumption for the first 3 weeks when the treatment with BTL had begun, but after the 4th week showed an increase in the food consumption (Fig. 95).

2.2.3.2. Methemoglobin

The DEN-DDS-BTL group had slightly higher methemoglobin levels than the DEN-DDS group after 2 weeks of DDS administration. After 2 weeks there was a steady decrease in the methemoglobin levels in the DEN-DDS and DEN-DDS-BTL groups, which persisted for the remaining period of observation (Fig. 96).

4.2.2.3.3. Liver weight

No significant difference in the relative liver weights of the DEN-DDS group and the DEN-DDS-BTL group were observed (Fig. 97).
4.2.2.3.4. Spleen weight

There was a significant increase (p<0.05) in the relative spleen weight of the DEN-DDS-BTL group as compared to the DEN-DDS group (Fig. 98).

As shown in table, the degree of hemosiderosis was similar in both the DEN-DDS-BTL and DEN-DDS groups.

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS-BTL</td>
<td>+++</td>
</tr>
</tbody>
</table>

4.2.2.3.5. Liver Glutathione levels

The total liver GSH levels were almost identical in both DEN-DDS-BTL and DEN-DDS groups (Fig. 99).

4.2.2.3.6. Liver preneoplastic foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 100 and 101.

4.2.2.3.6.1. Volume of foci/nodules

There was no difference in the volume of foci in the DEN-DDS-BTL group in comparison to the DEN-DDS group (Fig. 102).

4.2.2.3.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS-BTL group was slightly more than the DEN-DDS group (Fig. 103), however the increase was not statistically significant.
Fig. 94  Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot's Administration of promoter (and modulator) begins at week 2

Fig. 95  Showing average daily food consumption per rat in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot's Administration of promoter (and modulator) begins at week 2
Fig. 96 Showing weekly methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model Pitot’s

Fig. 97 Showing relative liver weights of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot’s
Fig. 98  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot's

* = p<0.05

Fig. 99  Showing liver glutathione levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot's
Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-PitotCFF; Image+MSVB6; 12-08-2002; CNJ-PSRM
File ID: 767
Rat: cr767
Liver wt.: 6.573

Marker Color:
- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.4473
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 100 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Pitot's model).

Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-BTL200-PitotCFF; Image+MSVB6; 12-15-2002; CNJ-PSRM
File ID: 761
Rat: CR761
Liver wt.: 7.453

Marker Color:
- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.6682
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 101 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-BTL group (Pitot's model).
Fig. 102 Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot’s.

Fig. 103 Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Pitot’s.
4.2.2.4. Effect of BTL on DDS Promoted Hepatocarcinogenesis in Ito Model

4.2.2.4.1. General health conditions (Cage side observations and body weight)

No detectable abnormality was observed in the health behaviour and activity in any of the rats of DEN-DDS and DEN-DDS-BTL groups.

Both the DEN-DDS and DEN-DDS-BTL groups showed a steady gain in body weight during the 8-weeks observation period (Fig. 104). However the gain was much less in the DEN-DDS-BTL group after partial hepatectomy as compared to the DEN-DDS group.

4.2.2.4.2. Methemoglobin

BTL administration along with DDS significantly (p<0.001) inhibited a rise in the methemoglobin concentration during the 2nd to 4th week period. The methemoglobin levels dropped in the DEN-DDS group after 4 weeks of DDS treatment and at the end of the study period there was no statistically significant difference between the methemoglobin levels in the two groups (Fig. 105).

4.2.2.4.3. Liver weights

The DEN-DDS-BTL group had a significantly lower (p<0.01) relative liver weight than the DEN-DDS group (Fig. 106).

4.2.2.4.4. Spleen weights

The DEN-DDS-BTL group had a lower relative spleen weight than the DEN-DDS group, however the decrease was not statistically significant (Fig. 107).

As shown in the table, the degree of hemosiderosis was lower in the DEN-DDS-BTL group than the DEN-DDS group.
<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS-BTL</td>
<td>+</td>
</tr>
</tbody>
</table>

4.2.2.4.5. Liver Glutathione levels

There was a statistically significant (p<0.05) decrease in the total liver GSH level in the DEN-DDS-BTL group compared to the DEN-DDS group (Fig. 108).

4.2.2.4.6. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 109 and 110.

4.2.2.4.6.1. Volume of foci/nodules

The DEN-DDS-BTL group showed a slightly reduced volume of foci than the DEN-DDS group (Fig. 111), but the difference was not statistically significant.

4.2.2.4.6.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-DDS-BTL group was slightly less than the DEN-DDS group (Fig. 112), however the decrease was not statistically significant.
Fig. 104 Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito’s. Administration of promoter (and modulator) begins at week 2. Partial hepatectomy.

Fig. 105 Showing weekly methemoglobin levels in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model Ito’s. 

*** = p<0.001
Fig. 106  Showing relative liver weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito's.  
** = p<0.01

Fig. 107  Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito's
Fig. 108 Showing liver glutathione levels of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito's.

* = p<0.05
Fig. 109 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Ito's model).

Fig. 110 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS-BTL group (Ito's model).
Fig. 111 Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito's.

Fig. 112 Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder (DEN-DDS-BTL) simultaneously with DDS. Model: Ito's.
4.2.2.5. Effect of BTL on AAF Promoted Hepatocarcinogenesis in Ito Model

4.2.2.5.1. General health conditions (Cage side observations and body weight)

No detectable abnormality was observed in the general health, behaviour and activity in the rats of DEN-AAF-BTL group. The rats belonging to the DEN-AAF group were weak and had an unkempt fur coat but were otherwise more or less active.

The DEN-AAF-BTL group showed a steady gain in weight whereas the DEN-AAF group showed a slight decrease in the body weight during the 8-weeks observation period (Fig. 113). However the gain was less in the DEN-AAF-BTL group rats from week 3 onwards, but later the gain was steady.

4.2.2.5.2. Liver weights

The DEN-AAF-BTL group and the DEN-AAF group showed almost similar relative liver weights (Fig. 114).

4.2.2.5.3. Spleen weights

The DEN-AAF-BTL group had a lower relative spleen weight than the DEN-AAF group; however this difference was not statistically significant (Fig. 115).

4.2.2.5.4. Liver Glutathione levels

There was a statistically significant (p<0.001) decrease in the total liver GSH levels in the DEN-AAF-BTL group in comparison to the DEN-AAF group (Fig. 116).
4.2.2.5.5. Preneoplastic liver foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 117 and 118.

- 4.2.2.5.5.1. Volume of foci/nodules

The DEN-AAF-BTL group showed slightly increased foci volume than the DEN-AAF group (Fig. 119), but the difference was not statistically significant.

- 4.2.2.5.5.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-AAF-BTL group was more than the DEN-AAF group (Fig. 120), although the difference was not statistically significant.
Fig. 113 Showing gain/loss in body weight in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito's. Administration of promoter (and modulator) begins at week 2, ▲ Partial hepatectomy.

Fig. 114 Showing relative liver weights in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito.
Fig. 115 Showing relative spleen weights in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito's.

Fig. 116 Showing liver glutathione levels in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito's

*** = p<0.001
**Fig. 117** Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-AAF group (Ito's model).

**Fig. 118** Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-AAF-BTL group (Ito's model).
Fig. 119 Showing liver foci volume in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito's.

Fig. 120 Showing number of foci/liver in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder (DEN-AAF-BTL) simultaneously with AAF. Model: Ito's.
4.2.2.6. Effect of BTL on PB Promoted Hepatocarcinogenesis in Ito Model

4.2.2.6.1. General health conditions (Cage side observations, body weight and food consumption)

No observable abnormality was observed in the general health, behaviour or activity in any of the rats of DEN-PB, DEN-PB-BTL and DEN-BTL groups.

Both the DEN-PB-BTL and DEN-PB groups showed a similar pattern of gain in body weight. A slight loss of weight after partial hepatectomy was observed in both the groups, but improved later during the remaining 5-weeks observation period. The DEN-BTL group lost weight after partial hepatectomy and after 4 weeks of BTL treatment, but gained weight by week 7 (Fig. 121).

4.2.2.6.2. Liver weights

The DEN-PB-BTL group and the DEN-PB group had almost similar relative liver weights (Fig. 122). The DEN-BTL group on the other hand, showed a significantly lower (p<0.05) relative liver weight compared to DEN-PB group.

4.2.2.6.3. Spleen weights

The DEN-PB-BTL, DEN-BTL and DEN-PB groups had almost similar relative spleen weights (Fig. 123).

4.2.2.6.4. Liver Glutathione levels

There was a decrease in the total liver GSH level in the DEN-PB-BTL group compared to the DEN-PB group. DEN-BTL group on the other hand, showed slightly higher liver GSH levels than the DEN-
PB group. However the differences in the groups were not statistically significant (Fig. 124).

4.2.2.6.5. Liver preneoplastic foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 125, 126 and 127.

4.2.2.6.5.1. Volume of foci/nodules

The DEN-PB-BTL group showed a slightly increased volume of foci over that found in DEN-PB group (Fig. 128), but the difference was not statistically significant. The DEN-BTL group had a highly reduced focal volume than the DEN-PB group (p<0.01).

4.2.2.6.5.2. Numerical density of foci/nodules in liver

The total number of foci/liver observed in the DEN-PB-BTL and DEN-BTL group were more than the DEN-PB group (Fig. 129), however the increase was not statistically significant.
Fig. 121 Showing gain/loss in body weight in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's Administration of promoter (and modulator) begins at week 2.

Fig. 122 Showing relative liver weights in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's

* = p<0.05
Fig. 123 Showing relative spleen weights in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's.

Fig. 124 Showing liver glutathione levels in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's.
Overlay of Tissue Images with 4 Markers.
Exp. ID: DEN-PB-ItoCFM; Image+MSVB6; 12-14-2002; CNJ-PSRM
File ID: 754
Rat: CR754
Liver wt.: 7.86

Marker Color:
1: S,
2: R, GGT
3: Y,
4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.4620
3: 0.0000
4: 0.0000

: Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 125 Plot picture of GGT stained liver section generated by Pitot’s program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-PB group (Ito’s model).

Overlay of Tissue Images with 4 Markers.
Exp. ID: Gr35DEN-PB-BTL200ItoCFM; Image+MSVB6; 11-25-2002; CNJ-PSRM
File ID: 741
Rat: CR741
Liver wt.: 9.5

Marker Color:
1: S,
2: R, GGT
3: Y,
4: G.

Tis. Area; sq cm
1: 0.0000
2: 3.3464
3: 0.0000
4: 0.0000

: Location of Anatomic Marker A, B. 1 cm Scale:

Fig. 126 Plot picture of GGT stained liver section generated by Pitot’s program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-PB-BTL group (Ito’s model).
Overlay of Tissue Images with 4 Markers.

Exp ID: Gr34DEN-BTL200ItoCFM; Image+MSVB6; 11-26-2002. CNJ-PSRM
File ID: 700
Rat: CR700
Liver wt.: 4.871

Marker Color:

- 1: S.
- 2: R. GGT
- 3: Y.
- 4: G.

Tis. Area: sq cm

1: 0.0000
2: 1.1871
3: 0.0000
4: 0.0000

Fig. 127 Plot picture of GGT stained liver section generated by Pitot’s program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-BTL group (Ito’s model).
Fig. 128 Showing liver foci volume in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's.

* = p<0.05

Fig. 129 Showing number of foci/liver in DEN-initiated and PB (DEN-PB) and BTL (DEN-BTL) -promoted animals treated with betel leaf powder (DEN-PB-BTL) simultaneously with PB. Model: Ito's.
4.2.2.7. Effect of BTL on the Initiation Stage of DDS Promoted Hepatocarcinogenesis in Ito Model

4.2.2.7.1. General health conditions (Cage side observations, body weight)

No detectable abnormality was observed in the general health status, behaviour and activity in DEN-DDS group. Rats of the BTL-DEN-DDS group were less active and had an unkempt fur.

The DEN-DDS group showed a better gain in body weight than the BTL-DEN-DDS group. The BTL-DEN-DDS group showed a loss in weight after partial hepatectomy but recovered in 2 weeks. The animals gained weight thereafter, but the gain was not as good as seen in DEN-DDS group (Fig. 130).

4.2.2.7.2. Liver weights

There was no significant difference in the relative liver weights of BTL-DEN-DDS and DEN-DDS groups (Fig. 131).

4.2.2.7.3. Spleen weights

The BTL-DEN-DDS group had a lower relative spleen weight than the DEN-DDS group; however the difference was not statistically significant (Fig. 132).

4.2.2.7.4. Liver Glutathione levels

There was a slight increase in the total liver GSH level in the BTL-DEN-DDS group compared to DEN-DDS group, but the increase was not statistically significant (Fig. 133).

4.2.2.7.5. Liver preneoplastic foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot’s program during analysis is shown in Figs. 134 and 135.
4.2.2.7.5.1. Volume of foci/nodules

The BTL-DEN-DDS group showed a significantly decreased (p<0.05) foci volume compared to DEN-DDS group (Fig. 136). The BTL-DEN-DDS group had 78.04% less foci volume than the DEN-DDS group.

4.2.2.7.5.2. Numerical density of foci/nodules in liver

The total number of foci/liver in the BTL-DEN-DDS group was slightly more than the DEN-DDS group (Fig. 137); the increase was however not statistically significant.
Fig. 130  Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS). Model: Ito's - Administration of promoter begins at week 2, - Partial hepatectomy.

Fig. 131  Showing relative liver weights of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS). Model: Ito's
Fig. 132 Showing relative spleen weights in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS). Model: Ito's.

![Relative Spleen Weight Graph](image)

Fig. 133 Showing liver glutathione levels of DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS). Model: Ito's.

![Liver Glutathione Level Graph](image)
Overlay of Tissue Images with 4 Markers.
Exp ID: DEN-DDS60-ItoCFM; Image+MSVB6; 12-15-2002; CNJ-PSRM
File ID: 6671
Rat: CR667
Liver wt.: 10.49g

Marker Color:
1: S.
2: R. GGT
3: Y.
4: G.

Tis. Area: sq cm
1: 0.0000
2: 3.1735
3: 0.0000
4: 0.0000

Fig. 134. Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Ito's model).

Overlay of Tissue Images with 4 Markers.
Exp ID: Gr46BTL-DEN60ItoCFM; Image+MSVB6; 11-26-2002; CNJ-PSRM
File ID: 8021
Rat: CR802
Liver wt.: 8.403

Marker Color:
1: S.
2: R. GGT
3: Y.
4: G.

Tis. Area: sq cm
1: 0.0000
2: 2.6648
3: 0.0000
4: 0.0000

Fig. 135. Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of BTL-DEN-DDS group (Ito's model).
Fig. 136 Showing liver foci volume in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS) Model: Ito’s

\[ * = p < 0.05 \]

Fig. 137 Showing number of foci/liver in DEN-initiated and DDS-promoted (DEN-DDS) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-DDS) Model: Ito’s
4.2.2.8. Effect of BTL on the Initiation Stage of AAF Promoted Hepatocarcinogenesis in Ito Model

4.2.2.8.1. General health conditions (Cage side observations, body weight)

The rats belonging to both the groups were sluggish and had unkempt fur.

Rats of DEN-AAF group did not gain weight almost throughout the observation period. Rather a loss in weight from week 3 to week 7 post-DEN was observed. In contrast, rats of BTL-DEN-AAF group showed a steady gain till week 3, but lost thereafter to almost reach their initial body weight by week 8. (Fig. 138).

4.2.2.8.2. Liver weights

The BTL-DEN-AAF group had as slightly lower relative liver weight compared to DEN-AAF group, but the difference was statistically not significant (Fig. 139).

4.2.2.8.3. Spleen weights

The relative spleen weights were almost similar in the BTL-DEN-AAF and DEN-AAF groups (Fig. 140).

4.2.2.8.4. Liver Glutathione levels

There was no statistically significant difference in the total liver GSH level between BTL-DEN-AAF group and the DEN-AAF group (Fig. 141).

4.2.2.8.5. Liver preneoplastic foci/nodules

A representative plot picture of the composite of montage of liver sections generated by the Pitot’s program during analysis is shown in Figs. 142 and 143.
4.2.8.5.1. *Volume of foci/nodules*

The BTL-DEN-AAF group showed slightly decreased foci volume compared to DEN-AAF group, but the decrease was statistically not significant (Fig. 144).

4.2.8.5.2. *Numerical density of foci/nodules in liver*

The total number of foci/liver observed in the BTL-DEN-AAF group was slightly more than the DEN-AAF group (Fig. 145), however the increase was statistically not significant.
Fig. 138 Showing gain/loss in body weight in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-AAF). Model: Ito's. Administration of promoter begins at week 2. Partial hepatectomy.
Fig. 140 Showing relative spleen weights in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-AAF). Model: Ito's.

Fig. 141 Showing liver glutathione levels in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-AAF). Model: Ito's.
Fig. 142 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-AAF group (Ito's model).

Fig 143 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of BTL-DEN-AAF group (Ito's model).
Fig. 144 Showing liver foci volume in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-AAF) Model: Ito's.

Fig. 145 Showing number of foci/liver in DEN-initiated and AAF-promoted (DEN-AAF) animals treated with betel leaf powder at the initiation stage along with DEN (BTL-DEN-AAF) Model: Ito's.
4.2.2.9. Effect of Bengal Gram on DDS Promoted Hepatocarcinogenesis in Pitot Model

4.2.2.9.1. General health conditions (Cage side observations, body weight and food consumption)

No detectable abnormality in behaviour or activity was observed in any rat belonging to the two groups.

The rats of both DEN-DDS (standard rat pellet only) and DEN-DDS (standard rat pellet + Bengal gram) gained weight more or less consistently throughout the observation period (Fig. 146). However, DEN-DDS (standard rat pellet + Bengal gram) rats gained weight better especially during week 8 to week 13 post-DEN.

This largely corresponds with the slightly better food consumption by these rats during this period (Fig. 147). The food consumption was not consistent in both the DEN-DDS (standard rat pellet + Bengal gram) and DEN-DDS (standard rat pellet diet only) groups. However, on an average DEN-DDS (standard rat pellet + Bengal gram) diet group consumed more food than the group fed the standard rat pellet diet only, especially from week 3-6 and week 10-14, post DEN.

4.2.2.9.2. Methemoglobin

Methemoglobin levels were largely identical in both the groups (DEN-DDS- standard rat pellet + Bengal gram) and (DEN-DDS – standard rat pellet only) throughout the observation period (Fig. 148).

4.2.2.9.3. Liver weights

The relative liver weight of the DEN-DDS (standard rat pellet only) group was slightly but significantly (p<0.05) higher than in the DEN-DDS (standard rat pellet + Bengal gram) group (Fig. 149).
4.2.2.9.4. **Spleen weights**

There was a significant increase (p<0.01) in the relative spleen weight in DEN-DDS (standard rat pellet only) group as compared to the DEN-DDS (standard rat pellet + Bengal gram) group (Fig. 150).

As shown in the table below, the degree of hemosiderosis was similar in both the groups.

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Degree of hemosiderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEN-DDS</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>DEN-DDS No Gram</td>
<td>+++</td>
</tr>
</tbody>
</table>

4.2.2.9.5. **Liver Glutathione levels**

The total liver GSH levels were significantly (p<0.01) higher in the DEN-DDS-pellet only group than the DEN-DDS (standard rat pellet + Bengal gram) group (Fig. 151).

4.2.2.9.6. **Liver preneoplastic foci/nodules**

A representative plot picture of the composite of montage of liver sections generated by the Pitot's program during analysis is shown in Figs. 152 and 153.

4.2.2.9.6.1. **Volume of foci/nodules**

The foci volume was more in DEN-DDS (standard rat pellet only) group than DEN-DDS (standard rat pellet + Bengal gram) group; the increase was statistically not significant (Fig. 154).
4.2.2.9.6.2. **Numerical density of foci/nodules in liver**

The total number of foci/liver observed in the DEN-DDS (standard rat pellet only) group was significantly ($p<0.05$) more than the DEN-DDS (standard rat pellet + Bengal gram) group (Fig. 155).
Fig. 146 Showing gain/loss in body weight in DEN-initiated and DDS-promoted (DEN-DDS) animals not fed with Bengal gram (DEN-DDS-No Gram) simultaneously with DDS. Model: Pitot's. Administration of promoter begins at week 2.

Fig. 147 Showing weekly food consumption in DEN-initiated and DDS-promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot's. Administration of promoter begins at week 2.
Fig. 148 Showing weekly changes in methemoglobin levels in DEN-initiated DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot's. * = p<0.05

Fig. 149 Showing relative liver weights of DEN-initiated and DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot’s. * = p<0.05
Fig. 150 Showing relative spleen weights of DEN-initiated and DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot’s

** = p<0.01

Fig. 151 Showing liver glutathione levels of DEN-initiated and DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot’s.

** = p < 0.01
Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-PitotCFF; Image: MSVB6; 12-08-2002; CNJ-PSRM
File ID: 757
Rat: cr757
Liver wt.: 6.673

Marker Color:

1: S.
2: R. GGT
3: Y.
4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.4473
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B

Fig. 152 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS group (Pitot's model).

Overlay of Tissue Images with 4 Markers.
Exp.ID: DEN-DDS50-NoGram-PitotCFF; Image: MSVB6; 12-12-2002; CNJ-PSRM
File ID: 795
Rat: CR796
Liver wt.: 8.198

Marker Color:

1: S.
2: R. GGT
3: Y.
4: G.

Tis. Area; sq cm
1: 0.0000
2: 2.2054
3: 0.0000
4: 0.0000

Location of Anatomic Marker A, B

Fig. 153 Plot picture of GGT stained liver section generated by Pitot's program showing transections of foci/nodules converted to circles of equivalent diameter with centroids. Representative section of DEN-DDS (No Gram) group (Pitot's model).
Fig. 154 Showing liver foci volume in DEN-initiated and DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot’s

Fig. 155 Showing number of foci/liver in DEN-initiated and DDS promoted (DEN-DDS) animals not fed Bengal gram in the diet (DEN-DDS No Gram) simultaneously with DDS. Model: Pitot’s

* = p<0.05