INTRODUCTION
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The dramatic advances in medicine in the last fifty years have stemmed in large part from extensive research to discover new effective medications for controlling and treating many types of diseases that afflict mankind. In the development of new drugs, an increasingly important aspect is to predict and assess any adverse effects that the product might have. Potential undesirable effects depend on the specific properties of the medication, including its biotransformation in the human body, the dose regimen, the age and sex of the patient and the general condition of the patient, especially in regard to nutritional or immunological status. The demonstration of drug safety involves assurance that major acute toxic effects will be avoided and evidence that long-term use will not cause chronic diseases, including cancer. Pharmaceuticals as with other chemicals have caused cancers in humans (Adamson and Sieber, 1981; Hoover and Fraumeni, 1981). A classic example is bladder cancer induced by chlorambazaine, a derivative of the known human carcinogen 2-naphthylamine, which was used in the treatment of polycythemia vera (Thiede and Christensen, 1969). The synthetic hormone diethylstilbestrol, used primarily for the maintenance of pregnancy has shown to cause vaginal adenocarcinomas in the offspring's of women receiving this drug during fetal embryogenesis (Herbst, et al. 1971).

Determination of carcinogenic potential of pharmaceuticals has therefore been a regulatory requirement in new drug development programs particularly in view of irreversible nature of cancer and the limited therapeutic repertoire currently available with us for the treatment of cancer. However the sufficiency of test strategies for identification and risk assessment of carcinogenic pharmaceuticals is dependent on our understanding of the carcinogenic process itself, which has seen phenomenal progress only in recent years.
Carcinogenesis is currently understood as a multistage process involving two operationally recognizable stages of pre-neoplasia, namely 'initiation' and 'promotion' before the lesions become autonomously growing neoplasms through progression (Roud and Kidd, 1941; Berenblum, 1941).

Initiation is a genetic event and irreversible in nature (Wyllie, 1987). Initiators are normally genotoxic/mutagenic and react with DNA to form DNA-adducts. DNA damage is repaired by various repair mechanisms present in the cell, such as the direct reversal of DNA damage, base excision repair, nucleotide excision repair, double-strand break repair and mis-match repair. When the rate of the occurrence of damage exceeds the capacity of the repair mechanisms the damage is fixed and gives rise to a mutation (Dragan, et al. 1994) which is inherited by the progeny of the cell. Several nitrosamines, aflatoxins, aromatic hydrocarbons and DNA alkylating drugs such as cyclophosphamide, melphalan, chlorambucil, nitrogen and uracil mustards are potent initiators of carcinogenesis.

The promotion stage is characterized by non-autonomous clonal expansion of the initiated cells without the necessity of further DNA damage. Diverse classes of pharmaceuticals that are not mutagenic/genotoxic act at the promotion stage and are classified as promoters or epigenetic carcinogens. Steroidal contraceptives, phenobarbitone, phorbal esters and peroxisome proliferators that include the hypolipidemic drugs clofibrate, ciprofibrate, fenofibrate, nafenopin, tibric acid, tiadenol and Wy14643 are some of the known promoters (Reddy and Lalwani, 1983). The mechanism by which these agents promote carcinogenesis is not well understood and the available data indicates that it is as diverse as the chemical classes the agents belong to. Promoters may alter gene expression related to growth regulation, gap junction inter cellular communication (Moennikes, et al. 2000). For a few agents such as the sex steroids, peroxisome proliferators, cyclosporine and tetradecanoylphorbol acetate (TPA), specific receptors have been classified (Kemp, etal. 1989). The androgen and estrogen receptors in the case of sex steroids, the peroxisome proliferator activated receptor (PPAR) for the
However, neither DDS nor its metabolites are mutagenic in the Ames assay (Peters, et al. 1978) or in the sister chromatid exchange assay (Dr. P. S. R. Murthy, unpublished observations). Interestingly, DDS is highly non-polar with a log $P$ value of 0.4383, a feature that is consistently found in non-mutagenic carcinogens (McCoy, et al. 1990). This may be due to their ability to interact with specific receptor sites or a longer residence time in the body.

Taken together, these evidences suggest that DDS is probably a promoter of carcinogenesis but no studies were conducted to date to establish this using appropriate mechanistic model. Keeping in view this paucity of information the International Agency for Research on Cancer concluded in 1987 that there is ‘insufficient’ evidence to classify the carcinogenic potential of DDS (IARC, 1987).

DDS has been found to cause agranulocytosis (Machet, et al. 1996), aplastic anaemia, hemolytic anaemia (Jollow, et al. 1995) methemoglobinemia and other blood dyscrasias, (Sczeremeta and Dohan, 1995) peripheral neuropathy and hepatic damage. A rare hypersensitivity reaction called “Dapsone Syndrome” may also occur in the first six weeks of DDS therapy in leprosy patients, and the symptoms include rash, fever, jaundice and eosinophilia (Tomecki, et al. 1981; Bocquet, 1995) The metabolites of DDS, rather than DDS itself are mainly responsible for these adverse effects.

In the body DDS is metabolized in the liver and the major metabolites of DDS are 4-Amino-4’-hydroxylaminodiphenylsulphone (DDS-NOH), 4-Acetylamino-4’-aminodiphenylsulphone (MADDS) and 4-Acetylamino-4’-hydroxylaminodiphenylsulphone (MADDS-NOH), (Fig. 1). The mechanism by which DDS induces hemolytic anemia and methemoglobinemia is thought to be via the interaction of N-hydroxyl DDS and mono-acetyl DDS with the hemoglobin molecule resulting in the oxidation of Fe$^{2+}$ to Fe$^{3+}$. This oxidized form of hemoglobin known as methaemoglobin has a reduced capacity to carry oxygen. This also renders the RBC susceptible to easy breakdown in the spleen and leads to accumulation of iron in the spleen. This overloading of effete RBCs and iron leads to splenomegaly and hemosiderosis (Graham, 1975).
Fig. 2 Products of dapsone metabolism
DDS NOH = 4-Amino-4-hydroxydimethylphenyl sulphone
MADDS = 4-Acetylamino-4-hydroxydimethylphenyl sulphone
Metabolism of dapsone (DDS)

**Primary Products**

**N-oxidation**

\[
\begin{align*}
\text{DDS} & \xrightarrow{N\text{-oxidation}} \text{DDS-NOH} \\
\text{MADDS} & \xrightarrow{N\text{-oxidation}} \text{MADDS-NOH}
\end{align*}
\]

**N-acetylation**

Fig. 2 Products of dapsone metabolism

- DDS-NOH = 4-Amino-4'-hydroxylaminodiphenylsulphone
- MADDS = 4-Acetylamino-4'-aminodiphenylsulphone
- MADDS-NOH = 4-Acetylamino-4'-hydroxylaminodiphenylsulphone
Currently concerted efforts are being made the world over to identify agents that can modulate toxicity and carcinogenicity favorably so as achieve ‘chemoprevention of toxicity’ and ‘chemoprevention of cancer’ respectively. These agents include pharmaceuticals, dietary constituents, phytochemicals, vitamins, etc. Of particular interest are some known pharmaceuticals such as cimetidine and tolbutamide, which in addition to their intended use could considerably suppress specific drug induced toxicity. Similarly the cancer chemopreventive/anti-carcinogenic potentials of several agents are currently either under evaluation in animal models or undergoing clinical trials.

Cimetidine {2-Cyano-1-methyl-3-[2-(5-methyl-imidazol-4-yl methylthio) ethyl] guanidine; CAS No. 51481-61-9; CMT} an H₂-receptor antagonist is widely used in the management of peptic ulcers. It is known to inhibit CYP1A2, CYP2C19, CYP2D6 and predominantly CYP3A4 isoforms of the CYP₄₅₀ microsomal enzymes, which are involved in DDS metabolism. Since DDS is largely metabolized by the CYP3A4 class of isoforms, coadministration of CMT with DDS has been found to reduce the hemotoxicity via inhibition of DDS metabolism by CYP3A4 (Rhodes, et al. 1995; Coleman, et al. 1992). No studies have been carried out till date on the suppression of DDS promoted hepatocarcinogenesis by CMT. Such information will not only elucidate the relationship between DDS toxicity and carcinogenicity but will provide new leads for designing ways of suppressing DDS promoted hepatocarcinogenesis.

Tolbutamide {1-Butyl 3-p tolylsulfonylurea; CAS No. 64-77-7; TLB}, a sulfonylurea is used in the treatment of Type II diabetes. Tolbutamide (TLB) has been reported to inhibit the metabolism of DDS in vitro (Winter, et al. 2000). TLB is a known inhibitor of CYP2C9, thus CYP2C9 is also involved in the metabolism of DDS. Whether TLB suppresses the promotion of hepatocarcinogenesis by DDS is not known and such information will facilitate the understanding the relationship between DDS metabolism, toxicity and carcinogenicity. It will also help in providing new leads for designing ways to suppress DDS promoted hepatocarcinogenicity.
Picroliv, an iridoid glycoside mixture containing Picroliv, picroside I and kutkoside is prepared from the roots and rhizomes of Picrorhiza kuroa belonging to the Scrophulariaceae family. Several beneficial effects of Picroliv have been reported. The glycosides present in picroliv have shown to exert anti-oxidant effects (Chander, et al. 1998). Picroliv has also been reported to be a hepatoprotective (Chander, et al. 1990) and inhibits the carcinogenesis process in the DEN initiated rat liver tumor model (Rajeshkumar, et al. 2000).

Piper betel (Piperaceae), commonly known as Betel is a native of southern India and Malaysia and is cultivated and consumed in western Pacific basin and southern Asia. Betel leaves are known to contain aromatic oil, minerals, glycosides, enzymes, vitamins, essential amino acids and tannins (Krishnakumar, et al. 2001). It is chewed along with areca nut and lime, the mixture known ad betel quid, has been reported to be a cause of oral cancer in many Asian countries. Contradictory reports are available over the cancer preventive/promoting properties of Betel (Piper betel) since it has been screened in different systems using either the betel quid or in some cases betel leaf alone. Aqueous extract of the leaves of Piper betle, given orally at different dose levels during the initiation phase of 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis in rats, inhibited the emergence of tumors (Rao, et al. 1985). Piper betle inflorescence contains hydroxychavicol a major phenolic component, and has been reported to be genotoxic (Lee-Chen, et al. 1996). Thus the effects of betel leaf on the process of carcinogenesis is not clear. Whether PLV or BTL suppresses the promotion of hepatocarcinogenesis by DDS is not known. Such information will facilitate the understanding the relationship between DDS metabolism, toxicity and carcinogenicity and also in promoting further the role of naturally occurring substances in chemoprevention. It will also help in providing new leads for designing ways to suppress DDS promoted hepatocarcinogenicity.

The two established and most widely used rodent hepatocarcinogenicity models for evaluating not only carcinogenic potential but also modulation potential of agents are those developed by Pitot et al. and Ito et al. The Pitot
assay uses female rats and is of 12-30 weeks duration while that of Ito uses male rats and is of 8 weeks duration. In both the assays appearance of preneoplastic liver foci/nodules expressing glutathione S-transferase (P) (GST-P) and gamma glutamyl transpeptidase (GGT) are taken as endpoint. The number of foci/liver and the volume of foci/ nodules are quantitated using stereological methods developed by Delesse (1848), Saltykov (1967) and Pugh, et al. (1983). The number of foci and volume of foci are indices of initiation and promotion, respectively.

The present study is undertaken with the following objectives
1. To determine the hepatocarcinogenic potential of DDS using short-term bioassays and to understand the stage specificity of the carcinogenic effect of DDS.
2. To investigate the effects of agents that interfere with DDS metabolism/disposition on the carcinogenic potential of DDS.
3. To test certain natural/synthetic products for their modulatory effects on carcinogenesis in the short-term models of hepatocarcinogenesis.
Plan of the study

The study was divided into two major parts. The first part of the study was to investigate the promotion potential of DDS while the second part was to investigate the modulation of DDS promoted carcinogenesis by different synthetic and natural agents.

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

1. Promotion of DEN initiated hepatocarcinogenesis by DDS

The promotion studies were further divided into two parts to compare the promotion potential of DDS with known standard promoters and secondly to investigate the dose response relationship (if any) of DDS in tumor promotion.

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

1.2 Dose response study on DDS in the Pitot model of hepatocarcinogenicity

2. Modulation of hepatocarcinogenesis

This study was divided 2 major parts, to study the modulation by CYP450 inhibitors and secondly by natural products.

2.1 Modulation by Cytochrome P450 Isoform inhibitors

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

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

2.1.3 Effect of TLB (a CYP 2C9 Inhibitor) on PB Promoted Hepatocarcinogenesis in Ito Model
2.2 Modulation by natural products

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

2.2.2 Effect of PLV on DDS Promoted Hepatocarcinogenesis in Ito Model

2.2.3 Effect of Betel Leaf (BTL) on DDS Promoted Hepatocarcinogenesis in Pitot Model

2.2.4 Effect of BTL on DDS Promoted Hepatocarcinogenesis in Ito Model

2.2.5 Effect of BTL on AAF Promoted Hepatocarcinogenesis in Ito Model

2.2.6 Effect of BTL on PB Promoted Hepatocarcinogenesis in Ito Model

2.2.7 Effect of BTL on the Initiation Stage of DDS Promoted Hepatocarcinogenesis in Ito Model

2.2.8 Effect of BTL on the Initiation Stage of AAF Promoted Hepatocarcinogenesis in Ito Model

2.2.9 Effect of Bengal Gram on DDS Promoted Hepatocarcinogenesis in Pitot Model