EFFECT OF LIPOSOME-ENCAPSULATED RADIOSENSITIZER (AK-2123) ON DEN INDUCED CANCER IN MICE

ABSTRACT

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SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
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ABSTRACT

Cancer is one of the major causes of death worldwide. It is caused by the progressive growth of the progeny of a single transformed cell, which is no longer responsive to normal growth controlling mechanisms. Therefore, curing cancer requires special strategy to selectively destroy all the malignant cells without damaging the normal cells. Conventional radiotherapeutic regimen often fails to cure cancer patients because they do not kill all malignant cells. Since, the interaction of radiation with matter is random, the radiation alone will damage both normal as well as transformed cells when the subject is irradiated. The damaged transformed cell may not survive and, thereby, cancer may be eliminated. However, there is a possibility that partially damaged normal cells may undergo transformation in due course of time and reestablish the disease.

The combination of radiomodulatory drugs with radiation has been considered to beneficial for radiotherapy of cancer. It has, indeed, improved the rate of cure of cancer and the outcome of treatment is better than radiation alone. The two different types of radiomodulatory drugs currently used in chemo-radiotherapy are: -

1. Radioprotective drugs that should essentially protect normal cells/tissues from the undesirable damage of radiation, thereby, paving way for application of higher doses of radiation for efficient killing of cancerous cells/tissues.
2. Radiosensitizing drugs that should, in principle, sensitize cancerous cells/ tissues so that they are killed efficiently even by relatively low dose of radiation, thereby, reducing the undesirable damages to normal cells/tissues.

However, the toxicity of drugs to other tissues and various other side effects have limited the application of chemo-radiotherapy in humans. In order to overcome these limitations of chemo-radiotherapy and to improve upon its therapeutic index, concept of drug delivery system has been tried in the last two decades. The concept is based on the fact that certain biologically acceptable carrier may be employed to deliver the drugs to biological target cells/tissues. Of several possible carriers, liposomes has been found to be promising and convenient because it's organized structure could accommodate drugs in the aqueous or lipid phase, depending on their solubility. Liposome is a lipid bilayer that folds back on itself creating a hollow sphere within which, it can enclose or entrap a variety of

substances. Structural versatility and biodegradable nature have made liposomes a more potent biological carrier system in drug delivery and drug targeting.

The work embodied in this thesis is an attempt to evaluate the whole body γ-radiation effect in the presence of AK-2123, a hypoxic cell radiosensitizer on tumor regression in mice. AK-2123 has widely been used in combination with a number of cancer therapies such as thermotherapy, chemo-therapy and radiotherapy.

Mice were chronically exposed to diethylnitrosamine, a potent hepatocarcinogen, for tumor induction. Cancer induction was studied by monitoring the marker enzymes activities, i.e. GGT and AChE, and the rates of nucleic acid synthesis. Histology and electrophoretic studies of surface membrane glycoproteins in liver were also carried out. Regression studies were carried out by administration of AK-2123 either in it's free or liposomal forms, in combination with whole body γ-irradiation 30 min. after the drug administration. The entrapment efficiency of liposomes for AK-2123 was determined using LASER Raman Spectroscopy. For the regression studies the same parameters employed in induction studies were used in addition to some haemopoietic parameters. The liposomal AK-2123 is envisaged to be tested *in vivo* for its radiosensitizing efficacy in normal and transformed system vis-à-vis the free form of AK-2123.

From the experiments conducted it was found that radiation treatment alone resulted in mass destruction of the cells. AK-2123 alone did not exihibit toxic effects on mice whether it was presented in it's free form or encapsulated in liposomes. However, a combination of these three factors i.e. radiation, AK-2123 and liposomes, produced satisfactory results. Liposome encapsulated AK-2123 was found to be more efficient radiomodulator compared to it's free form. The main points emerging from the work embodied in the thesis are: -

- Chronic exposure to NDEA combined with hepatectomy induced cellular transformations in the liver of Swiss albino mice, as substantiated by the pronounced alterations in the activities of the marker enzymes and the synthetic indices of the nucleic acids.

- NDEA treatment resulted in a distinct change in the nature of the hepatocytes such as the variations in the cell shape and size, appearance of more densely stained nuclei and multinucleated cells as elucidated in the histological studies.
- Upon NDEA exposure, the liver glycoproteins exhibited differential expressions. Some proteins were over expressed while others were under expressed as compared to their normal counterparts as revealed by the electrophoretic study.
- Radiation alone inflicted damage to both the transformed and normal cells, as reflected by the marked decline in the activities of the marker enzymes and synthetic indices of the nucleic acids.
- Liposomes obtained by the reverse phase evaporation method were relatively large in size and most of them were unilamellar.
- About 40% entrapment efficiency of AK-2123 into liposomes was achieved as determined by LASER Raman spectroscopy.
- AK-2123 alone, whether present in free or liposome encapsulated forms, had no toxic effect on the liver at a concentration of 200 mg/kg body weight.
- Liposomes retained the properties of the entrapped AK-2123. It's selective migration to the liver ensured that a major proportion of the drug reached the liver intact, as is evident from the levels of the activities of the marker enzymes and nucleic acid synthetic indices, which were in the proximity of the normal levels. This is further supported by the observation that AK-2123 when encapsulated in liposomes showed lesser suppressive effects on the haemopoietic parameters studied, as compared to it's free form.
- AK-2123 in the oxic conditions of irradiation exhibited lesser radiosensitizing effects as compared to the hypoxic mode of irradiation thereby, affording radioprotective effects. It is seen from the fact that none of the transformed groups of animals administered with either free or liposome encapsulated AK-2123 and followed by exposure to radiation in oxygenated conditions, regained their actual normal levels of enzyme activities and nucleic acids' synthetic indices, nor fell below the normal levels.
- Overall, AK-2123 encapsulated in liposomes afforded better radiomodulation than free AK-2123.

Thus, it is seen that treatment of cancer with a combination of radiation, a radiomodifier and a drug delivery system, opens a wide scope for exploitation for the

improvement of existing cancer therapies and the chances of curing and combating the menace of the dreadful cancer.

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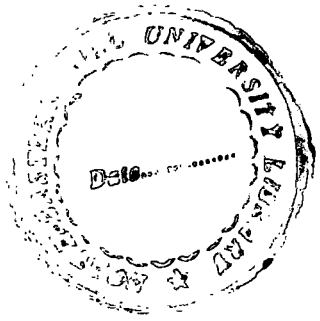

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In Memory Of My Father

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I, **Chowphi Chen Rapthap**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University or Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor Of Philosophy in Biochemistry.

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1 INTRODUCTION

Cancer has been known since human societies first learnt to record their activities. It was well known to the ancient Egyptians and to succeeding civilizations. The term Cancer covers a large range of neoplastic diseases for which a multiplicity of biological, physical and chemical causes are known or suspected.

Cancer is increasingly recognized as a disorder resulting from abnormal regulation. It usually appears as a tumor (swelling) made up of a mass of cells, and the visible tumor is the end result of a whole series of changes which may have taken many years to develop. One important characteristic of cancer cells is that they are no longer responsive to normal growth controlling mechanisms. Since there are almost certainly many different growth control factors involved, the altered cells may still respond to some but not to others. Strangely enough, for reasons still unknown, rapid cell growth itself is not necessarily associated with an increased risk of tumor development, e.g. tumors of the small intestine are very rare[84]. Cancer strikes humans of all ages and a wide variety of organs are affected. Although most cancers develop late in life so that until the expectation of life began to increase from the middle of the 19th century onwards, the number of people surviving into the 'cancer age' was relatively small. The incidence of cancer increases with age, so that as people live longer, more are likely to develop the disease. Cancer poses a serious threat to human society and is now one of the major causes of death. Apart from individual sufferings, the economic burden to society is immense. For all these reasons, its control or even better, prevention are important. But cancer research has an even wider significance[84].

A large number of potentially carcinogenic agents are present in our diet and environment. Approximately, 70% to 90% of all cancers in human have been attributed to environmental causes[127]. These carcinogenic agents have been broadly classified into three categories- biological, physical and chemical carcinogens. Biological agents, predominantly viruses, cause a variety of cancers, physical carcinogens comprise mainly of ionizing radiations and radioactive substances; while chemicals now recognized as causing or modifying cancer, include a very extensive range of chemical classes, inorganic as well as organic, as compared to the time when chemical carcinogens meant only a few polycyclic hydrocarbons and azo dyes and little else[127].

Carcinogenesis is a multistage process. The application of a carcinogen does not lead to the immediate production of tumor. It involves three steps, namely, initiation, promotion and progression.

Initiation is a very rapid and irreversible event. Once the initial change has taken place the initiated cells may persist for a considerable time, perhaps the lifespan of an individual. The most likely site for the primary event is in the genetic material, DNA, although there are other possibilities. The carcinogen is thought to damage or destroy specific genes probably in the stem cell population of the tissue involved[30]. The fixation of damage caused by a carcinogen can take place if the cell replicates while DNA damage is persistent. Permanent alteration in the genome can be produced by several possible ways; the mispairing of bases leading to point mutations; errors in replication yielding frame–shift mutations; transpositions resulting in codon rearrangement; and combinations of these alterations in sequential steps. Codon rearrangement may involve sequences known as oncogenes, which are emerging as critical gene sequences for transformations[164, 20]. In the case of interactions with the mitotic apparatus, chromosomal mutations and aneuploidy could result. All these alterations generate a permanently abnormal cell with an altered genotype and distinct phenotypes[168].

Initiated cells remain latent until acted upon by promoting agents. Many of these 'transformed' cells may not grow at all or grow very slowly while others may acquire the capacity to proliferate beyond tissue constraints to form neoplasms. It is at this stage that the influence of growth appears. Promoting agents are not carcinogenic in themselves but they induce initiated cells to divide. Many agents will induce cell division, but only promoters will induce tumor development, so that although cell growth is necessary for tumor development, there must be other factors involved. Partial hepatectomy can in fact act as promoting stimulus in rat liver carcinogenesis, and tumor transformation arise in the regenerated liver after an initiating, prehepatectomy treatment with a carcinogen. The suggestion is that promoting agents may interfere with the process of differentiation, which normally takes place when cells move from the dividing stem cell population into functioning and usually non-dividing cells. Even though these growth promoting stimuli are acting on the cells, they may still be sensitive to the normal growth inhibiting factors in the body so that the final outcome depends on the balance between the factors and the extend of the changes in the initiated cells. This explains why preneoplastic or even apparently fully

transformed tumors can be found but do not appear to be growing or sometimes even regress[84].

The term, progression, is usually reserved for the process by which cells of a benign or malignant tumor acquire more and more aberrant characteristics- the bad to worse principle of tumor evolution[169]. During this step a series of changes take place after a tissue cell is initiated, but the rate at which this occurs depends on changes in the cell and on changes in the host. Most carcinogens are very highly reactive and when they react with DNA in the affected cell they usually damage other sites, as well as the relatively few which are thought to control neoplastic transformation. Thus, the same agent may produce tumors in a given organ, which differ greatly from each other, depending on specific genes, which have been altered or lost. At one extreme, if only the 'transforming' sites have been altered, the resulting tumor cells will still retain much of the normal differentiated structure and function of the cell from which they have arisen. If the genes responsible for the normal structure are more severely damaged, the resulting tumor cells have fewer normal properties. At the other extreme, the cells may have lost almost all the normal properties of the cells from which they have arisen. The loss of normal characteristics is known as dedifferentiation or anaplasia. Some cells may have lost their specific structural characteristic but still retain differentiated biochemical characters, and others may still appear structurally differentiated but have lost many functional attributes[84].

The whole sequence of events in the process of tumor formation is almost certainly a consequence of gene changes, although gene expression may be influenced by the host. The discovery that oncogenes of tumor producing viruses (protooncogene) in normal, as well as in some tumor cells has led to intensive research into the relationship of these genes to normal and tumor growth and development. These genes have been localized to specific chromosomes and some to sites of chromosome abnormalities in tumors. Much speculation now centers on the question whether the initiation, progression and maintenance of some tumors depends on over-expression through gene amplification or inappropriate expression (i.e. wrong time) of normal genes or whether mutations in a critical region of a gene is necessary. A possible hypothesis is that, a mutation may be necessary for the initiation event but that some or all of the later stages may depend on over – or inappropriate expression[84]. The expression of such genes on tumor cells however, differs from the expression on normal cells. For instance, they are present at high levels on tumor cells and

in trace amounts on normal cells, they are usually distributed over the cell membrane, or they are expressed at an inappropriate phase of oncogenesis.

A variety of genes are involved in adaptive response of the cells. Particularly, signal transduction pathway regulated by p38 MARK and PKC families, plays an important role in this response. Among the many genetic alterations, which have been documented in human tumors, mutation of the p53 gene is one of the most common[88, 67]. p53 has a major role in maintaining normal cellular phenotype, it is involved in adaptive response and the cells lacking this gene do not inhibit adaptive response[75]. Many functions have been attributed to p53, among the most important regarding tumorigenesis are transcriptional regulation, cell cycle control and involvement in signalling for apoptosis. The role of p53 in carcinogenesis has been investigated and reported that loss or mutation of p53 is rate limiting for carcinogenesis[78]. One of the best studied aspects of p53 is it's role in apoptosis. Apoptosis is another important radiation response of the cells after low dose of radiation[28]. Apoptosis is thought to function as a safe guard of the body by killing possible deleterious cells carrying DNA damage. Cells lacking functional p53 are defective in apoptosis following several stimuli, including exposure to ionizing radiation[92, 34, 104, 91]. This finding explains, at least in part, the observation that p53 mutations lead to elevated radiation resistance in haemopoietic cells[87]. Not only do cells carrying mutant p53 fail to die following DNA damage but they also have been observed to be defective in G_1 phase cell cycle arrest[77, 82, 89, 171]. The observation that p53 gives rise to defective G_1 arrest led to the hypothesis that p53 occupies an important position in the signalling pathway(s) between DNA damage and cellular consequences such as death by apoptosis, cell cycle arrest and repair of damage[85]. In support of this argument, p53 has been found to influence genome stability as assessed by permissiveness for DNA amplification induced by drug treatments[89, 171]. It has been reported that the predominant *in vivo* function of p53 in haemopoietic tissue lies in the late phase of the cell cycle and is associated with the G_2/M checkpoint and chromosome segregation. Thus, in addition to the well established G_1 checkpoint function, p53 functions at other stages of the cell cycle[25]. Other evidence supports the suggestion that p53 has a late cell cycle function[145]. Therefore, cancer is prevented by apoptosis, and suppression of apoptosis is one of the steps to carcinogenesis. There is increasing evidence that loss of 'tumor suppressor genes' is also implicated in the generation of the more common human cancers, which have no clear hereditary pattern. Since in principle it is easier to inactivate a gene functionally by mutation or deletion than to activate a proto-oncogene in a specific

way, the tumor suppressor genes may constitute important targets for carcinogenic chemicals at some stage of tumorigenesis[169].

Tumors are not homogeneous; a developed tumor usually consists of a mixed population of cells, which may differ, in structure, function, growth potential, resistance to drugs or X- rays and the ability to invade and metastasize. Although progression is usually towards greater malignancy, this is not invariably so. There are a number of cases – unfortunately small – in which rapidly growing tumors have ceased to grow or even disappeared completely and this behavior still remains an enigma. Another major and unexplained area is concerned with the time scale of carcinogenesis. The latent period between initiation and the appearance of tumors is one of the least understood aspects of tumor development. Yet another unexplained fact is that only a small number of cells 'initiated' by a carcinogen will eventually produce tumors – perhaps only one or two from many millions of treated cells[84]. These non-homogeneous properties and nature of tumors are posing a great hindrance in cancer therapy.

1.1 CHEMICAL CARCINOGENS

Chemical carcinogens are reactive intermediates, as electrophilic reactants or radical cations, which can interact with cellular macromolecules. Chemical carcinogens can be defined operationally by their ability to increase the occurrence of neoplasms[165]. Four types of increased response are generally adopted as evidence:

1. The development of types of neoplasms not seen in control.
2. An increased incidence of the types of neoplasms seen in control.
3. The occurrence of neoplasms earlier than in control.
4. Increased multiplicity of neoplasms in individual animals.

Chemical carcinogens are a highly diverse collection of chemical substances, including organic and inorganic chemicals, solid–state materials, hormones and immunosuppressants. For the chemicals that appear to operate as neoplasm enhancers or promoters, the designation "carcinogen" is unfortunate, because the agents probably cannot initiate the process leading to cancer[165, 147].

Classes of chemical carcinogens

Chemical carcinogens can be classified into two major categories - Genotoxic and Epigenetic[165]. Chemical carcinogens that interact with, and alter DNA are classified as genotoxic. This category contains the "classic" organic carcinogens that are electrophilic reactants either in their present form or after metabolism. Most likely, DNA alteration is the key event in the initiation of carcinogenicity by these compounds. These agents operate primarily in the sequence of neoplastic conversion, but may also promote carcinogenic process through reactive or other metabolites. The effects of inorganic chemicals on the fidelity of DNA polymerases suggest that carcinogenic metals might yield abnormal DNA by a distinct mechanism involving alteration of the fidelity of DNA polymerases[173]. Genotoxic carcinogens, probably because of their effects on DNA, are occasionally effective after a single exposure and frequently carcinogenic at subtoxic doses. They act in a cumulative manner and act together with other DNA – reactive carcinogens having the same organotropism. They usually produce neoplasms in more than one target organ and have a short latent period[168]. The second broad category, designated as, epigenetic agents, comprises those chemicals for which no evidence exists of direct interaction with genetic material, but which produce another biological effect that could be the basis for their individual carcinogenicity. This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants and promoters. Promoters by definition do not have intrinsic properties of altering genetic apparatus. Possible mechanisms for epigenetic effects include chronic tissue injury, hormonal imbalance, immunologic effects or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens. Thus, epigenetic agents operate primarily in the sequence of neoplastic development[168].

In contrast to genotoxic agents, epigenetic agents are a highly diverse group; generalizations are difficult. Nevertheless, epigenetic agents usually affect only selected organ systems where they produce physiological perturbations that usually require a long latent period to show their effects. In some cases, such as promoters, carcinogenic effects occur mainly with high and sustained levels of exposure that lead to prolonged physiological abnormalities, hormonal imbalances and tissue injuries[168].

Some important chemical carcinogens are listed in Table 1[127].

Table 1 Chemical Carcinogens.

Class	Compound
Polycylic aromatic hydrocarbons	Benzo[a]pyrene, Dimethylbenzanthracene.
Aromatic amines	2-Acetylaminofluorine, N-methyl-4-aminoazobenzene(MAB).
Nitrosamines	Dimethylnitrosamine, Diethylnitrosamine
Various drugs	Alkylating agents (e.g. Cyclophosphamide), Diethylstilbestrol.
Naturally occurring compounds	Dactinomycin, Aflatoxin B_1.
Inorganic compounds	Arsenic, Asbestos, Beryllium, Cadmium, Chromium.

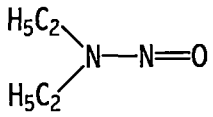
1.2 N- NITROSODIETHYLAMINE (DEN)

The chemical carcinogen DEN is a derivative of nitrosamine. The carcinogenic property of DEN was indicted soon after the carcinogenic activity of N-Nitrosodimethylamine (NDMA) was first described by Magee and Barnes[96] in 1956 and which was confirmed by Schmähl and Preussman[135]. The early data clearly indicated the marked biological activity and particularly the potent carcinogenic effects of N-Nitroso compounds, namely nitrosamines and nitrosamides. Pharmacokinetics and pharmacodynamics of simple dialkylnitrosamines were studied by Heath, Dutton and Magee[65, 66], Associated with these preliminary investigations was the demonstration in 1962 of *in vivo* alkylation by nitrosamines of cellular constituents, specifically proteins[97, 98] and nucleic acids[167]. Nitrosamines in general have been reported to be complete carcinogens and can act as initiators and promoters[169].

The liver is the main target organ for malignant transformation and tumor induction in rat by homologous series of symmetrical dialkylnitrosamines. In mice liver, kidney as

well as lung and the haemopoietic system are the predominant target organs. By and large, organ specificity of symmetrical dialkylnitrosamines seems to be independent of the route of administration of the carcinogen[121].

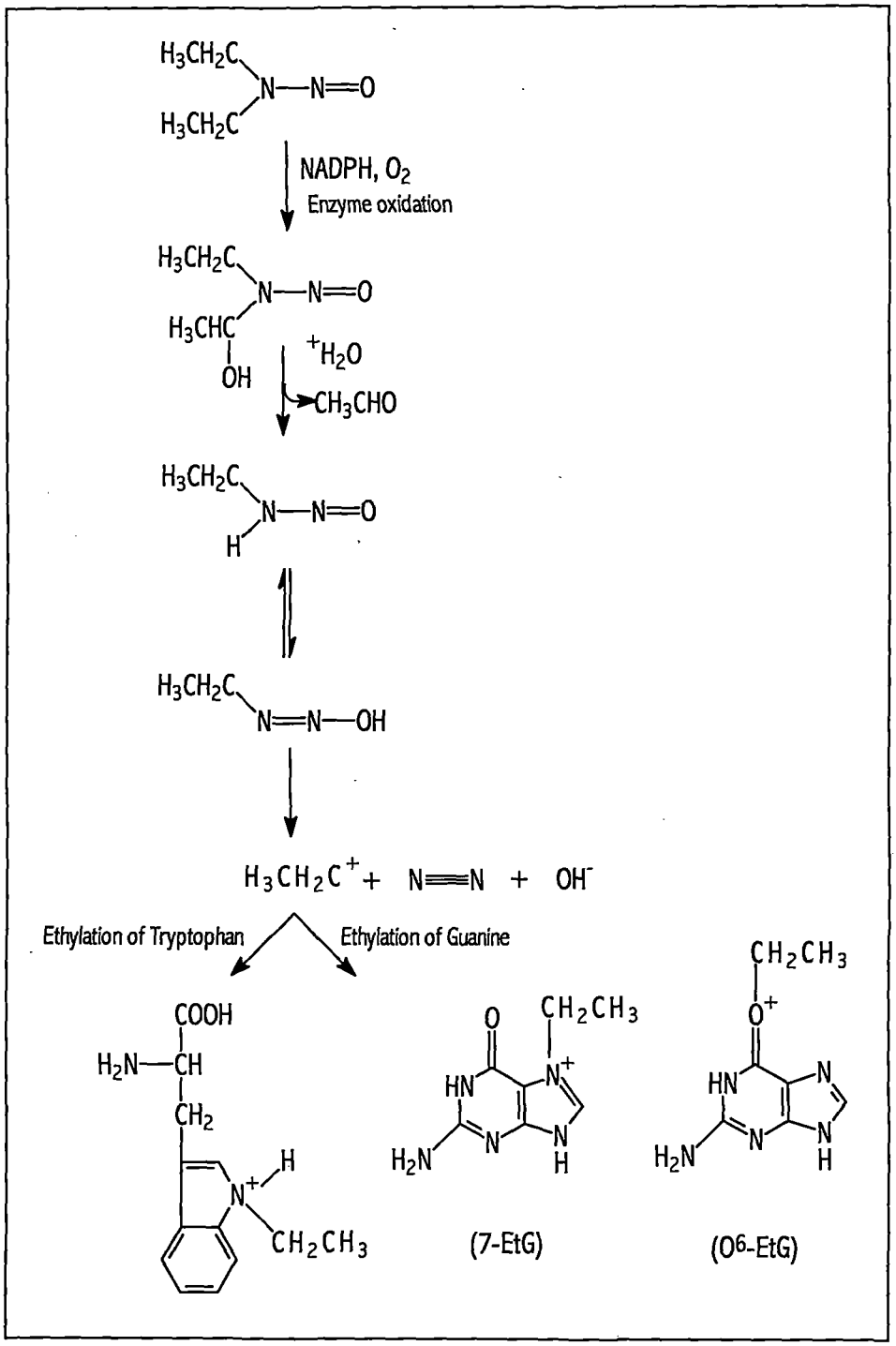
DEN is a chemically stable compound and it produces usually systemic cancer distant from the site of it's application. It has the following chemical structure, and it's lethal dose, LD_{50}, is 280 mg/kg.



DEN in it's parent form is not an active carcinogen. It requires metabolic activation to generate the active moiety, which is probably an electrophilic ethylating agent, as the ultimate carcinogen[100, 101].

Metabolic Activation of DEN

α–Hydroxydiethylnitrosamine has been proposed to be a proximate carcinogen, and is a highly reactive species. This reactive intermediate is then easily hydrolyzed to form acetaldehyde and a tautomeric diazohydroxide. Loss of nitrogen then leads to the formation of a carbonium species, which is an electrophilic ethylating moeity that would act as the ultimate carcinogen[64, 108]. The reactive electrophilic species is capable of being bound to the nucleophilic centers of cellular constituents and especially to DNA[105, 138]. Since DNA, RNA and proteins may have more than one nucleophilic centers, therefore it remains to be seen how does ethylation occurs, and which are the preferred nucleophilic centers. This is explicable by mechanistic organic chemistry. Alkylating agents that react by a unimolecular substitution mechanism (S_N1) react more extensively with O atoms in DNA, including the O-6 of guanine and O atoms of phosphate groups, relative to alkylating agents that alkylate via a bimolecular substitution pathway (S_N2). The likely reaction pathway is determined by the sensitivity of the alkylating species to the strength of the nucleophile with which it reacts; this property[86] is expressed by the Swain-Scott factor *s*. Alkylating agents having relatively high Swain-Scott factor (e.g. methyl methane sulfonate) react more exclusively at the major nucleophilic centre, the N-7 of guanine, and are less likely to attack O sites.



Ethylation of DNA and Carcinogenesis

Alkylation at certain sites in DNA has been well characterized and these sites include nitrogen atoms at positions 7 and 3 of guanine, positions 1, 3 and 7 of adenine, and position 3 of thymine and cytosine and oxygen atoms at position 6 of guanine, position 4 of thymine

and in phosphate groups[116, 86]. Approximately 50% of the total ethyl groups bound to DNA were present as ethylphosphotriester; ethylated bases were present in the following order: 7-EtG > O^6-EtG > O^2-ethylthymine =3-EtA > O^4-ethylthymine = O^2ethylcytidine[116, 86]. Ethylation of DNA suggested that base pairing does not significantly hinder alkylations of oxygens. The base-paired oxygens have an electron pair not involved in hydrogen bonding and thus available for reaction[140, 21]. Patterns for ethylation of RNA differ only slightly from those observed in DNA; their chemistry is similar. Thus, distinctions already made between S_N1 and S_N2 reactions for DNA apply equally well to RNA.

In vivo studies on malignant transformation have shown that ethylation at the O^6-position of guanine not only disrupts normal base-pairing but may also affect the stability of nucleic acid structure. This inference has been drawn from thermodynamic studies[103]. More insight into the biological impact of O^6-ethylation, not only in terms of miscoding but also in reference to the enzymology of DNA repair are anticipated[12]. Examination of the possible relationship between ethylation of DNA and carcinogenesis was based primarily on the knowledge that ethylation at each of the various sites within nucleic acids destabilizes the macromolecular structure to varying degrees. In both DNA and RNA, ethylation at the O^6-position of guanine is thought to destabilize the glycosyl bond[99]. Three independent studies have reported that O^6-alkylguanine, in different organs, could be correlated with tumor development. These three studies have common features: after a single dose of carcinogen, the persistence of O^6-alkylguanine in DNA from the organ where tumors would be anticipated was contrasted with persistence of O^6-alkylguanine in DNA from liver (a non-target organ); comparison was also made of the persistence of 7-alkylguanine in the DNA from both organs. No evidence of organ-specific differences in the rate of loss of 7-alkylguanine was observed[80, 115].

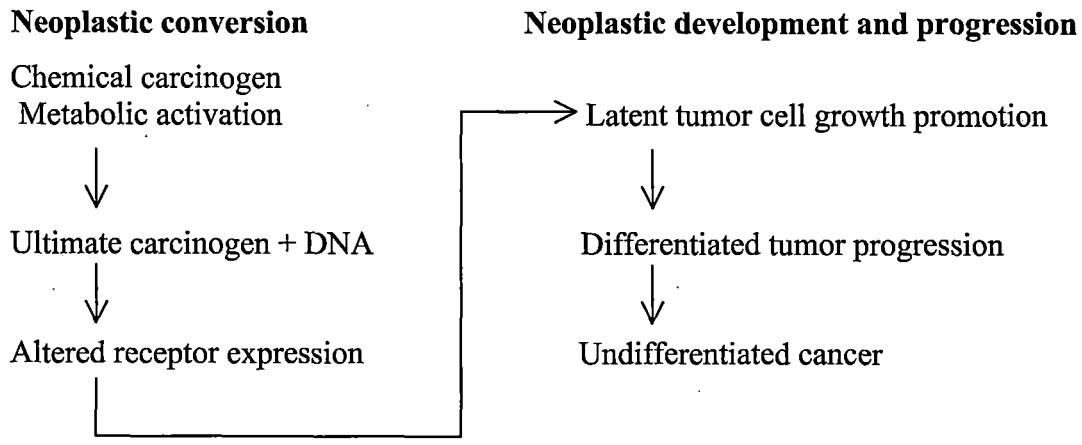
Experiments originally initiated to examine hepatocarcinogenic activity of dialkylnitrosamines in relation to persistence of O^6-alkylguanine in rat liver DNA have provided data that are paradoxical but that do not exclude involvement of this modified base in the carcinogenic process. As pointed out by Pegg, even the induced repair activity is unable to eliminate all the O^6-alkylguanine produced in hepatic DNA; at least some of the promutagenic base persists[117]. Multiple dose and chronic exposure to dialkylnitrosamines resulted in the increase of the O^6-alkylG/O^7alkylG ratio of cerebral DNA from 0.28 to 0.68. The impact of this report was considerable in establishing the significance of O^6-alkylation

for carcinogenesis and the findings complemented and extended with those made with single dose regimes[115].

The effect of exposure to DEN on chromatin includes not only ethylation of DNA but ethylation of chromatin protein as well, as is evident from the critical role that chromatin proteins especially, non-histones, may play in control of gene expression[144, 24]. Alterations in thermal stability of rat liver chromatin that was induced *in vivo* by DEN was reported by Stewart and Farber[146]. In addition changes in the biophysical properties of chromatin, as well as variation in the electrophoretic mobility of the specific chromatin proteins may be caused by ethylation of the proteins and possibly the nucleic acid components of chromatin. It has been suggested that the conformation of DNA *in vivo* may influence the site of alkylation[126].

The various modified bases in DNA constitute only a single type of carcinogen-induced change in genetic material; structural change in DNA associated with elimination of adducts *in vivo* may have equal biological significance[121].

The chemical carcinogenic process is a multistage process involving many reactions, which can be grouped into sequences (shown in scheme I). In the first sequence, the normal cell is converted to a neoplastic cell. In the second sequence, the neoplastic cell develops into an overt neoplasm. Chemicals are involved in a diverse way in both sequences[168].



Scheme I: Sequence of complex events during chemical carcinogenesis.

1.3 MONITORING CANCER

Generally, cancer is associated with the abnormal production of enzymes, proteins and hormones; and among the increasing number of biochemical markers for malignant cells, enzymes play an important role in cancer detection. Activity changes in the enzyme patterns (deficiency or activation) are common findings in neoplastic tissues. Several tumor marker enzymes are available, but for liver carcinogenesis, GGT assumes a significant importance because of it's unique properties. Two tumor marker enzymes i.e. GGT and AChE, which were used in the present study, shall be discussed here.

Gamma-Glutamyl transpeptidase (GGT; EC 2.3.2.2), is a plasma membrane glycoprotein, bound on the excellular side of the plasma membrane, the saccharide moiety of which exhibits a high heterogeneity and organ specificity and is involved in kinetic properties[124]. GGT remains unique as the only protease that can cleave intact glutathione[37]. It is stated that GGT catalyzes three types of reactions i.e. hydrolysis, transpeptidation and auto-transpeptidation. GGT catalyzes the hydrolysis of the gamma-glutamyl compounds and the split gamma-glutamyl moiety can be further transferred by GGT either to free amino acids or to small peptides in a transpeptidation reaction, or returned to other donor molecules in an auto-transpeptidation reaction[7]. Furthermore, inhibition by the acceptor substrate occurs[14]. A 'ping-pong bi-bi' mechanism is proposed for transpeptidation and auto-transpeptidation reactions[19]. GGT plays a key role in the gamma-glutamyl cycle, a transport system of neutral amino acids or small peptides across the cell membranes[7, 3].

In liver, GGT has been recognized as a positive marker for hepatocytes that have undergone malignant transformation[23]. The link between GGT and liver carcinogenesis was discovered in 1972 by Fiala and co-workers when they reported elevated levels of the enzyme in livers of rats fed with the hepatocarcinogen, 3'-methyl-4-dimethylaminoazobenzene, and also in transplantable, chemically induced rat hepatomas. Spontaneous or experimentally induced hepatocellular carcinoma in rats and mice show marked elevation of GGT activity when compared to normal liver. A unique property about this enzyme is that the liver of adult rats and mice show very low, or negligible GGT activity, while livers of fetal rats and mice show very high GGT activity, which however rapidly drops to low adult level a few days after birth. However, during the course of carcinogenesis, this enzyme is strikingly activated[22, 113]. Thus, it serves as a relevant and

excellent tumor marker. Elevated levels of GGT during hepatocarcinogenesis are found not only in the liver of rats but in livers of other species as well. Human hepatocellular carcinomas also show increased levels of GGT[55]. This particular tumor marker enzyme had been utilized in the present study.

Generally, a single marker is not sufficiently useful and thus, in this study another marker enzyme, AChE was utilized. AChE (EC 3.1.1.7) a serine hydrolase, first described by David Nachmansohn (1938), is a membrane bound enzyme which plays a key role in cholinergic neurotransmission. AChE rapidly hydrolyzes the acetylcholine to acetate and choline, and so it efficiently terminates the chemical impulse, thereby setting the basis for rapid, repetitive responses and enabling the re-uptake (and recycling) of choline[13].

1.4 CANCER THERAPY

Conventional chemotherapeutic or radiotherapeutic regimens often fail to cure patients because they do not kill all malignant cells. One reason is that the dose needed to eradicate cancer is so large that patient's own tissues would be severely damaged. The usefulness of monoclonal antibodies and antibody conjugates for therapy is also limited by the extend to which they cross-react with, and harm normal tissues. Active specific immunotherapy with antigenic tumor cells (rendered non-tumorigenic by e.g., irradiation) or extracted tumor-associated antigens is another approach for improved systemic cancer therapy[125, 128]. The development of megavoltage X-ray and Cobalt-60 gamma- ray machines has made dramatic improvements in cancer treatment. In actual radiotherapeutic practice, the total dose is not chosen on the basis of tumor control. It is based primarily on the maximum dose tolerated by normal tissues, as determined by prior experience, with the aim of optimizing tumor control. Normal tissue tolerance is improved by using radiation with better dose localization characteristics, this in turn, make it possible to increase the dose to the tumor without exceeding normal tissue tolerance[95]. Proton beams have been extensively used for radiotherapy and radiosurgery[94]. The results suggest that postoperative proton therapy is the best form of current treatment for tumors.

Radiotherapy is the most widely used non-surgical method in the treatment of cancers. However, the clinical outcome is far from satisfying. The major obstacle for effective eradication of established metastases is the non-uniformity and non-homogeneous

nature of cells that constitute multiple cell population in tumors, with diverse biological heterogeneity in growth rate, karyotype, cell surface proteins, antigenicity, immunogenicity, marker enzymes; sensitivity to various drugs and ability to metastasize. The heterogeneous nature of malignant neoplasms and the continuous evolution of variant cells imply that the successful therapy of disseminated cancer must include a modality that can overcome these obstacles i.e. biological heterogeneity in metastases and the emergence of treatment – resistant tumor cells. The high intra and inter heterogeneity even in tumors of the same entity, same clinical staging and same histopathological grading, indicates that regions with high expressed parameters like vascularization, cell proliferation, oxygen rich areas will be found in the immediate neighbourhood to regions where these parameters are very little expressed. For such situations, the use of hypoxic cell sensitizers could be very promising if the sensitizer can be distributed over the whole tumor in a comparatively high concentration. In addition, the oxygenation of tumors plays a very important role in the treatment of tumors and therefore, many efforts have been taken to improve the oxygen state of the cells under treatment and to sensitize the more radio resistant hypoxic cells within tumors[150]. Thus, combination of radiomodulatory drugs with radiation has been considered to be beneficial for cancer therapy; it has indeed improved cancer treatment and has been shown to be far better than radiation alone.

Two different types of radiomodulatory drugs currently used in chemo-radiotherapy are: -

1. *Radioprotective drugs*. Radioprotective drugs should essentially protect normal cells/tissues from the undesirable damage of radiation and thereby, paving way for application of higher doses of radiation for efficient killing of cancerous cells and tissues.
2. *Radiosensitizing drugs*. Radiosensitizing drugs should in principle sensitize cancerous cells and tissues so that they are killed even by low doses of radiation and thereby, reducing the undesirable damages on normal cells and tissues.

But several of these radiomodulatory drugs proved to be hazardous to normal cells and tissues, for instance, their toxicity to other tissues and various other adverse side effects have limited and crippled the application of chemo-radiotherapy, which otherwise looks very promising.

1.5 LIPOSOMES

To overcome the limitations of chemo-radiotherapy and to improve upon its therapeutic index, a search for methods for rendering agents tumor specific was launched. This search led to the concept of drug delivery system, which involved the experimental used of a wide array of carriers with varying degrees of tumor specificities. Non-specific carriers include cells, liposomes and polymeric drug formulations[60, 120]. Structural versatility, innocuous nature and easy biodegradability, in addition to other properties, have made liposome a more potent biological carrier in drug delivery and drug targeting.

Liposomes are lipid vesicles which are formed when a lipid bilayer folds back on itself to form a hollow sphere, within which, it can enclose or entrap a variety of substances in addition to lipid soluble and lipid bound membranes. Spontaneous formation of liposomes occurs when a combination of certain lipids is dispersed throughout an aqueous solution[54]. Materials dissolved in the aqueous solution become trapped in the enclosed aqueous compartments, which form in an alternating, concentric fashion with the lipid bilayers. In addition, lipid-soluble materials may be incorporated in the formed liposomes if added to the lipids forming the bilayers structures. These properties make liposomes ideal carriers of drugs, enzymes and other biologically important compounds[47, 38].

The components of the formed liposomes, the nature of the material entrapped in the aqueous compartment and the size of the structures all contribute to the initial degree of entrapment of the aqueous material and the stability of the preparation[54, 72]. The biological distribution of the entrapped materials follows that of the liposome carrier. Phagocytosis of small diameter liposomes by the Kupffer cells of the liver occurs within minutes after intravenous injection[48, 33, 123]. The phagocytic removal of liposomes by the reticuloendothelial system hinders their use as drug carriers to afford specific delivery of medically important agent to sites of actions other than the reticuloendothelial system. However, this property has been used to obtain diagnostic information about the liver and spleen and may lead to the use of liposomes as a delivery system for radiopharmaceuticals[46]. The extend to which various tissues participate in the uptake of liposome-entrapped agent requires that (a) the agent is firmly associated with liposomes during it's transport so that the tissue distribution pattern obtained reflects that of the carrier, (b) the agent persists within the tissues for a time sufficiently long so as to allow bulk

elimination of liposomes from the blood[50]. The uptake of liposomes is size-dependent, it was reported that uptake in the liver and spleen was reduced by diminishing the size of liposomes, while uptake by tissues kidneys, lungs, skeletal muscles, brain was enhanced. This could be attributed to the slower rate of elimination[123] of smaller liposomes from blood (due in turn to a lesser hepatic and splenic involvement) which would allow a larger number of such liposomes to undergo transcapillary passage, escape the liver and spleen and reach the alternative tissues[51]. As hepatic and splenic uptake was found to diminish by decreasing liposomal size, it is conceivable that interference by these tissues would be further reduced with liposomes of even smaller average size or with uniformly small (but not necessarily monolamellar) liposomes. Other changes in physical properties of liposomes such as membrane fluidity and surface did not alter the uptake of liposomal encapsulated species by the tumor tissues. The recent *in vitro* investigation of Weissmann[53] and his coworkers have indicated that it is possible to enhance the uptake of liposomal entrapped enzyme into cells by modifying the liposome surface.

The preposition that liposomes[5, 53] could serve as carriers of drugs in cancer chemotherapy has attracted considerable interest[50]. Work with model systems has already shown that a liposome-associated drug can be more efficient in prolonging the survival of tumor-bearing animals[40], can exhibit intensified action[122], escape immature inactivation[62] and also act on cells otherwise resistant to the free drug[38]. Successful application of liposomes in cancer treatment however will require efficient transport of drugs by liposomes into the malignant area in the body. Various reports suggest that such drug transport may be related to the size[123, 51] and charge[54, 45] of the liposomes, their penetration of vascular barriers[156], the nature of the liposome-drug association[61, 71], the extend of liver and spleen participation in liposome uptake[50], the characteristics of the target malignant cells[10], the mode of liposome-cell interaction[50, 38] and also the possibility of targeting liposomes to the areas in need of treatment[49].

Most attempts to target liposomes to solid tumors have been disappointing because the phospholipid vesicles are unable to reach organ parenchyma and are rapidly bound to and endocytosed by phagocytic cells. However, taking advantage of this natural fate of liposomes to target (albeit passively) immunomodulators of cells of the mononuclear phagocyte system, Fidler and colleagues were able to activate these cells to the tumoricidal and virocidal state[114]. Successful application of liposomes in cancer chemotherapy will

probably require in addition to the appropriate adjustments of the carrier's size and charge some method for improving it's localization in the diseased area. The direction of liposomes to specific target cells *in vivo* can be achieved by altering the surface structure of liposomes. Immunopotentiation has been established both for antigens exposed on liposomal outer surfaces and for antigens encapsulated within the liposomes[114]. Systematic administration of liposomes containing various immunomodulators have been shown to bring about regression of lymph node, lung and liver metastasis, and dramatic prophylaxis of viral infections in rodents[114]. The development of a doxorubicin-liposome product to be used in treatment of cancer has met with recent success in targeted drug delivery to specific sites and tissues, and also in the reduction of toxicity and undesirable side effects of drugs. The liposome has been shown to quite significantly reduce the cardiovascular effects of the drug[143].

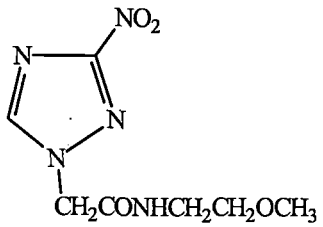
Theoretically, liposomes solve many problems of an antineoplastic or radiomodulators drug delivery. Antibiotic, antineoplastic, antimalarial, antifungal and anti-inflammatory agents have been found to be more effective when administered encapsulated in liposomes[32, 59]. Insoluble or poorly soluble drugs can be administered intravenously. Drugs having serious dose-limiting toxic effects on organs can be packaged in liposomes to avoid exposure to these organs. Similarly, high peak drug concentrations can be reduced by liposome formulation resulting in slow drug release. Some drugs are metabolized rapidly and their period of effectiveness is relatively short. Liposome by enclosing the drugs in it's hollow sphere delays it's metabolic alteration, preserving the encapsulated species in it's active form for a longer time, thereby, ensuring that maximum proportion of the drugs to be targeted or delivered, reaches it's target tissues with minimal or no alteration of the biological, chemical and physical properties of the drugs[6, 52].

At present, there are several methods of preparing liposomes, each having their own advantages and disadvantages. Some of these methods are as follows- Sonication method[4], Ethanol injection method[19], French Press method[18], Ether infusion method[39], Detergent dialysis method[76], Reverse phase evaporation (REV) method[152] and Rehydration dehydration method[79]. One of the major advantages of the REV is the relatively high entrapment efficiency for water-soluble materials. However, this method may not be suitable for encapsulation of proteins and nucleic acids since the method involves organic solvent and sonication that may lead to denaturation of the solute molecules.

Liposomes may differ in their dimensions, compositions (different phospholipids and cholesterol contents), charge (resulting from the charge of the composing phospholipids), and structure (multilamellar liposomes consisting of two or more concentric bilayers, separated by aqueous compartments, or unilamellar liposomes consisting of only one phospholipid bilayer surrounding one aqueous compartment). The *in vivo* distribution of liposomes is strongly influenced by their characteristics. Because liposomes are biodegradable and may be composed of nontoxic and immunologically inert phospholipids, they have been suggested as promising carriers for haptens, antigens and other drugs. The versatile nature of liposomes provides a distinct advantage for formulations tailored to specific pharmacological goals and present unique clinical and experimental opportunities. It may be possible to prepare liposomes with high degree of tissue specificity so that drugs and perhaps even enzyme replacement can be carried out with this technique. Evidence demonstrates that the systematic activation of macrophages by immunomodulators encapsulated in liposomes can provide a successful approach to the eradication of established cancer metastasis[123]. Hence, the prospects of drug delivery through liposomes have taken a great leap forward over the last few years from serendipity to targeting.

1.6 RADIOMODULATORY DRUG AK-2123

AK-2123, N- (2'-methoxyethyl)-2-(3"-Nitro-1"-triazolyl) acetamide, a nitrotriazole derivative, was proposed as a hypoxic cell radiosensitizer by the Kyoto University group 985). It has a molecular structure as shown: -



Physical properties

1. Melting point	: 126.5°C - 127°C
2. Octanol water partition coefficient:	: 0.14

3. Solubility of AK-2123 in saline solution :

Temperature (°C)	20	30	40	50	60
Solubility (%)	2.22	3.83	5.16	7.06	10.20

4. One-electron reduction potential: -1.12V vs. Ag/Ag^{+} (measured in N,N-dimethylformamide).

Physiological activity *in vivo*

(1) AK-2123 is less (1/2) toxic than Miso.
(2) AK-2123 has dose modifying factors of 1.41 and 1.28 for the i.v. and p.o. systems respectively.
(3) It exhibits thermosensitizing activity.
(4) It exhibits chemosensitizing activity for CDDP (Cisplatin).
(5) Induction of anti neoplastic activity by continuous low dose administration.
(6) Enhancement of the suppression of metastasis by CPM (Cyclophosphamide).
(7) Enhancement of efficacy of the combined modalities of (a) radio-thermotherapy (b) chemo-thermotherapy (c) thermotherapy.

In addition to these properties and effects, in vivo administration of AK-2123 also shows the following effects, each playing a significant role in cancer therapy involving AK-2123,

(1) It inhibits phosphorylation of glucose in tumors and in normal tissues.
(2) It inhibits tumor blood flow.
(3) It decreases tumor pH.
(4) It exhibits heat sensitizing effect in tumor thereby, inducing enhancement of hyperthermia.

Physiological activity *in vitro*

(1) AK-2123 is 1/5 less toxic and has lower mutagenicity than Miso[157].
(2) The chemosensitizing effect of AK-2123 has 1.8 times higher cytoxicity on tumor cells than Miso[68].

(3) The chemosensitizing activity of AK-2123 (100 μM) on the cell killing activity[68] of ADM showed a dose modifying factor of about 1.6.

(4) It exhibits 2.9 times selective inhibition activity to tumor cells

(5) The radiosensitivity of AK-2123 was compared with Miso (5mM/dm^{-3}) for V79-379A under both aerobic and hypoxic conditions[70].

Contacting with drug	Enhancement ratio	
	AK-2123	Miso
"**Oxic** conditions"		
- during irradiation	1.01	1.02
- during irradiation followed by 24 h aerobic incubation after aerobic irradiation	1.30	1.13
"**Hypoxic** conditions"		
- during irradiation	1.85	1.94
- during irradiation followed by 24 h aerobic incubation after hypoxic irradiation	2.40	2.12

AK-2123 exhibits lower (0.6) hypoxic radiosensitizing activity than Miso but higher (1.1) toxicity included enhancement ratio (by 24 h contacting with cell after irradiation*) in vitro.*

The radiomodifying properties of AK-2123 on tumor is mainly attributed to it's effect on the morphology of the vascular system and on the rheology of blood of the malignant tissues. The difference in response of the malignant and normal cells to the effect of AK-2123 lies in the nature of their vasculature. The vascular beds of the normal cells appear to be more resistant not only to AK-2123 but to hyperthermia as well, than the vascular beds of malignant cells. Kunigita, N. *et al.,* have shown that there was an apparent difference in the blood flow of tumor between AK-2123 administered and non-administered control mice. On the other hand, there was a similar change in the blood flow of normal mice regardless of AK-2123 administration. This observation indicates that the tumor vasculature is much more sensitive to AK-2123 than that of the normal cells[83]. This difference in the morphological features of the vessels of tumor cells and normal cells arises

from the fact that during angiogenesis, the hastily formed capillaries in tumor are made of single – layered endothelial cells without an external coat of elastic basement membrane, making them more fragile than the normal vessels and hence, facilitating metastasis. The capillary wall is lined in part by tumor cells between the gaps of endothelial cells. Therefore, the tumor cells are often in direct contact with circulating blood. Proliferation of these malignant cells into the lumen of the capillaries will obstruct blood flow and deteriorate the blood flow as the tumor grows. Tumor blood vessels are usually twisted, sharply bent with coil like features and are extremely dilated. In fact, it is suggested that the tumor capillaries are maximally dilated to meet the demand for nutrients by the continuously proliferating tumor cells[29].

It has long been known that the microenvironment of cancerous tissues is intrinsically acidic ralative to normal tissues, most probably due to the preponderance of glycolytic metabolism accompanied by the formation of lactic acid. AK-2123 inhibits the phosphorylation of glucose to glucose-6-phosphate[148]. This further brings down the tumor pH and thereby, enhancing the acidic condition in cancerous tissues. By this inhibition of phosphorylation, glucose cannot be used efficiently through glycolytic pathways and inhibition of energy supply takes place. Therefore, there is a significant increase in the levels of glucose and lactate while the pyruvate level decreases. These changes in lactate and pyruvate levels induce a remarkable increase of the lactate/pyruvate ratio. This metabolic situation of high lactate level upon AK-2123 administration leads apparently to very strong acidifications resulting in the drop of pH in the micro milieu of tumors, which is very damaging to the cell survival[129, 149]. This intrinsically acidic microenvironment highly favours the increase in the rigidity of the coursing RBC membranes[161]. The increased adherence of leucocytes to vascular walls, particularly venules, further obstructs the passage of the deformed and highly rigid RBC and thereby plug the capillaries and slows down the blood flow in tumors.

The quantitative changes of the relative radiosensitivity of malignant tumors and normal tissues as a function of oxygen tension (pO_2) have been impressively demonstrated by Vaupel[160]. Tumor cells in microenvironment with pO_2 values below those found in venous blood will have less than maximum radiation sensitivity. At an oxygen tension (pO_2) of 3 to 4 mm Hg, the radiosenstizing effect is only half-minimal compared to well oxygenated tissue. It has been shown that the median O_2 partial pressure of malignant tumor

is frequently lower than that of normal tissues. That means, if the status of tumor oxygenation would persist during the radiation treatment there would be no chance to cure a tumor without damaging heavily the adjacent normal tissue. Since the studies by van Putten and Kallman[158], it is known that tumors can reoxygenate especially during fractionated irradiation. The extend and rapidity of reoxygenation is extremely variable and impossible to predict[163]. The individual changes in tumor oxygenation during different treatments are supported by morphological studies on vascular injuries in the R1H tumor during radiation or radiation-hyperthermia treatment[175]. It has been reported that morphological changes of the blood vessels and connective network in the tumor periphery were not detected during the first two weeks of fractionated irradiation, but the combined treatment of radiation with AK-2123 and hyperthermia, damage the blood vessels and extra vasation occurs, many vessels get occluded and in some cases, the vascular network gets very disorganized that it appears like it is no longer present[176]. These morphological changes brought upon by AK-2123 i.e. breakdown of vascular system, vascular occlusion, vascular stasis, inhibition of blood flow and fall in blood pressure, result in the fall of oxygen partial pressure (pO_2) thereby, increasing the hypoxic cell fraction and their response to radiation. Therefore, besides acting as a hypoxic cell radiosensitizer, AK-2123 also increases the degree of hypoxic cell fraction (through severe restriction of blood flow) and consequently enlarging the proportion of cells on which it has it's hypoxic radiosensitizing effect. Thus, there occurs a strong interaction between vascularization, supply of nutrients and glucose to the tumor tissue, metabolism of glucose through glycolysis to lactate and cell killing.

Cell proliferation is also affected by the same parameters upon AK-2123 administration. It has been shown in cultures of human melanoma cells that a decrease of the pH which coincides with an increase of lactate causes a strong formation of quiescent S-phase cells. These quiescent S-phase cells show a DNA content, which is in agreement with the S-phase DNA content. However, DNA synthesis takes place no longer in these cells[174].

It is clear that blood plays an important role in the therapy of cancer involving AK-2123. Overall, the retardation of blood flow in tumors is partly due to the combined results of (a) swelling of endothelial cells (b) lysis of endothelial cells and tumor cells in the capillary wall accompanied by leakage of blood (c) sticking of leucocytes to vessels walls and (d) increase in rigidity of RBCs and viscosity of blood[29].

The effects of AK-2123 can be summed up as follows: –

1. AK-2123, like other hypoxic cell radiosensitizers, inhibit phosphorylation of glucose in tumors and in normal tissues. Cytotoxic effects are dependent on these phenomena.
2. Increase turnover of metabolism. If nutrient supply is reduced, energy depletion occurs and this can lead to breakdown of many metabolic processes.
3. Blood flow reduction causes lactate accumulation and hence, a decrease of pH in tumor, fall in pO_2 in tumor. These changes have a feedback on blood flow, which is further reduced.
4. Decreases nutrients supply with a decrease of pH and energy depletion or inhibition of the use of energy, increases cell death and reduce intracellular recovery processes. These metabolic changes lead to a decrease of cell proliferation, cell death and necrotic processes are induced and tumor regression is supported[151].

Although AK-2123 has been widely reported as a hypoxic cell radiosensitizer, however, a report by Hebbar and George[132] suggested that AK-2123 also exhibits radioprotective effects in well-oxygenated systems and in aerobic conditions of irradiation. By assessing radiation-induced lipid peroxidation in erythrocyte ghost membrane in the absence or presence of AK-2123, they found that AK-2123 alone did not exert any significant effect, but it markedly reduced the radiation-induced lipid peroxidation when present at all concentrations during oxygenated conditions of irradiation. Radiation induced normal tissue damage namely, muscle lipid peroxidation was also diminished following administration of the drug *in vivo* into Swiss mice. These observations suggested that under aerobic conditions AK-2123 protects against radiation-induced membrane oxidative damage. Therefore, this led Hebbar and George to claim that this was the first report demonstrating unequivocally that AK-2123 affords remarkable radioprotection against γ-ray induced membrane peroxidative damage both *in vitro* and *in vivo* if present during aerobic conditions of irradiation. This indicates that this compound has a dual function as a hypoxic cell radiosensitizer and also as a radioprotector against radiation-induced membrane peroxidative damage in well-oxygenated systems, which is invaluable in the radiotherapy of cancer[132]. This agrees with the finding of Mitsuhashi and his group (1998) who, when assessing the sensitizing effects of AK-2123 on radiations, cisplatin and hyperthermia found that there was no enhancing effect of AK-2123 on these parameters under aerobic

conditions *in vitro* [112]. In addition, reports are also available about the antimetastatic effect[81] of AK-2123 and it was suggested that this effect is related atleast partially, to the inhibition of the active calcium transport. While Shchepetkin[69] reported that inhibition of the ion transport of neutrophils by AK-2123 led to the inhibition of the priming effect of ionizing radiation on neutrophils.

Thus, AK-2123 not only has hypoxic cell radiosensitizing property, it also exhibits chemosensitizing properties, antimetastatic effects and it inhibits membrane ion transport system. It has also been reported to behave as a radioprotector in aerobic conditions of irradiation. Therefore, AK-2123 possesses multimodal properties, which can be exploited and utilized effectively in the war against cancer.

The threat and menace of cancer is rapidly spreading and is looming large in the society of mankind. It is now assuming an alarming mortality rate. It's evasive nature to the host's defense system and to the existing therapies only aggravates the already existing serious and grave problem related to this disease. An antidote against cancer is an urgent need if the gravity of the problem is to be eased at all. Realizing the potent properties and effects of radiation, the availability of the easily manipulated and other highly useful benefits of a drug delivery system and the vast scope that AK-2123 offers for exploitation in finding out means for improving cancer therapy and curbing down the incidence and spread of cancer, the piece of work embodied in this thesis had been proposed. The study is an attempt to evaluate the whole body gamma irradiation effect in presence of AK-2123, a radiomodifier, on cancer regression in mice. With this in mind, the following aims and objectives were conceived: -

1. Induction of cancer in mice by hepatocarcinogen, N-Nitrosodiethylamine (DEN).
2. Regression of cancer following γ- ray irradiation.
3. Effects of radiomodulatory drug on radiation induced cancer regression.
4. Effectiveness of radiomodulatory drug when delivered through liposomes.

2 MATERIALS AND METHODS

2.1 CHEMICALS

All the chemicals used were of analytical grade. The various chemicals were obtained from different sources as listed below: -

Nitrotriazole derivative **AK-2123** was a generous gift from Dr. V. T. Kagiya, Japan.

Sigma Chemicals: N-Nitrosodiethylamine (DEN), Glycylglycine, Bovine serum albumin (BSA), Trizma Base (Tris[hydroxymethyl]aminomethane), L-γ-glutamyl-p-nitroanilide, [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB), Acetylcholine chloride, Coomassie Brilliant Blue G-250, Trypan Blue, Collagenase, Sodium azide, Cholesterol, Dithiothreitol (DTT). Dicetyl Phosphate (DCP), DL- α-Phosphatidylcholine, Dipalmitoyl (DPPC), DL- α-Phosphatidylcholine, Dimyristoyl (DMPC), Fetal calf serum, L-glutamine, L-arginine, L-asparagine monohydrate, Ethylenediaminetetraacetic acid (EDTA), Triton X-100 (t-Octylphenoxypolyethoxyethanol).

Boehringer Mannheim: 5-Bromo-2'-deoxy-urudine Labeling and Detection Kit III, Penicillin-Streptomycin.

Qualigens: Sucrose, Trichloroacetic acid (TCA), Potassium ferricyanide $K_3Fe(CN)_6$, Potassium cyanide (KCN), Orthophosphoric acid (H_3PO_4), Sodium dihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$), di-sodium hydrogen ortho-phosphate (Na_2HPO_4), Sodium hydroxide flakes (NaOH), Xylene, D.P.X., Formaldehyde solution (HCHO), Methanol (CH_3OH), Eosin, Haematoxylin, Perchloric acid ($HClO_4$) 70%, Chloroform ($CHCl_3$), Glycerol, Glacial acetic acid (CH_3COOH).

Hi Media: Dulbecco's Modified Eagle Medium Base (DMEM), Potassium hydrogen phosphate (monobasic) KH_2PO_4, Potassium hydrogen phosphate (dibasic) K_2HPO_4.

Merck: Ammonium sulphate $(NH_4)_2SO_4$, Citric acid-1-hydrate($C_6H_8O_7.H_2O$), Sodium bicarbonate($NaHCO_3$), Hydrochloric acid (HCl).

BDH: Sodium chloride (NaCl), Paraffin Wax with ceresin.

Bengal Chemicals & Pharmaceuticals Ltd.: Ethanol (C_2H_5OH) 90%.

CDH: Picric acid.

Loba Chemie: Gower's Reagent (RBC diluting fluid).

s.d. fine-Chem Ltd.: WBC Diluting fluid.

Spectrochem: Scintillation fluid (Cocktail 'T').

Radioactive chemical: Uridine-5-T Tritium ([^{3}H]-U) from **BARC, India.**

Miscellaneous: Nitrogen gas ($N_2 \uparrow$).

2.2 INSTRUMENTS

Homogenizer: Remi Motors type RQ - 127 A, H.P 1/8, R.P.M. 8000.

Centrifuge: (i) Remi C 24, (ii) Beckman Optima™ TL Ultracentrifuge and (iii) Spinwin model MC - 01.

Spectrophotometer: Hitachi Model U-2001 UV/Vis Spectrophotometer.

Raman Spectrometer: 1403 Double Monochromator, Spex.

Liquid Scintillation Counters: (i) Beckman LS 1801 and (ii) System1400™ Wallac 1409.

Electrophoresis Unit: PhastSystem™ Pharmacia Biotech.

ELISA: Multiskan MS India Serial RS-232 C.

Microscope: (i) Carl Zeiss JENA 30 - G0603 and (ii) Leitz Dialux 20.

Microtomy: Weswox Rotary Microtome.

Aspirator and Rotary vacuum evaporator: EYELA A-3S and type N-1 respectively.

Miscellaneous Equipments: Neubauer hemacytometer, Water Bath SB-35, Syringes, Hamilton syringes, Dialysis tubings, Whatman filter papers, Whatman glassmicrofibre filters (GF/C 25 mm & 2 μ), Whatman filter units (0.45 μm), Parafilm M etc.

2.3 ANIMALS

Swiss Albino mice (Balb/c), bred by random inbreeding, were used for the entire course of study. The animals were maintained on basal diet *ad libitum* and housed in plastic cages in a temperature controlled animal room (21 ± 2°C) with a 12 h light and dark cycle.

2.4 INDUCTION OF CANCER BY DEN ADMINISTRATION

Healthy 6-8 weeks old mice were administered a large dose of aqueous preparation of DEN (150 mg/kg body weight) intraperitoneally, and the animals were allowed to recover for two weeks. The recovered mice were subjected to partial hepatectomy (50%) and within 24 h of partial hepatectomy, another intraperitoneal dose of DEN (100 mg/kg body weight) was administered, and the animals were allowed to recover for a week. After the recovery week, weekly intravenous administration of DEN (25 mg/kg body weight) was carried out for a period of three months. A 10% C_2H_5OH oral administration was initiated one week after the second dose of DEN following partial hepatectomy, and this continued till the day of sacrifice. Sham-treated, age–matched normal mice served as controls.

The following tumour markers were used in monitoring the induction of cancer: -

A) *Marker Enzymes*

1. Gamma-Glutamyl transpeptidase (GGT)
2. Acetylcholine esterase (AChE)

B) Nucleic Acids synthetic indices

1. RNA synthesis assay by Uridine-5-Tritium [^{3}H] incorporation
2. DNA synthesis assay by 5-Bromo-2'-deoxy-Uridine (BrdU) incorporation

In addition to these markers, *histological* and *electrophoretic* studies were also carried out.

2.5 TISSUE PREPARATION FOR ENZYMATIC ASSAYS

Experimental and normal mice were killed by cervical dislocation and the liver was quickly excised and removed, rinsed in chilled normal saline (0.9% NaCl), blotted dry and weighed. A 20% homogenate was prepared in chilled 0.32 M sucrose solution. The homogenate was solubilized in 0.5% Triton X-100 (1:1) and then centrifuged at 20,000 × *g* at 4°C for 30 min. The resulting supernatant containing soluble GGT and AChE was used for their assays and protein estimations.

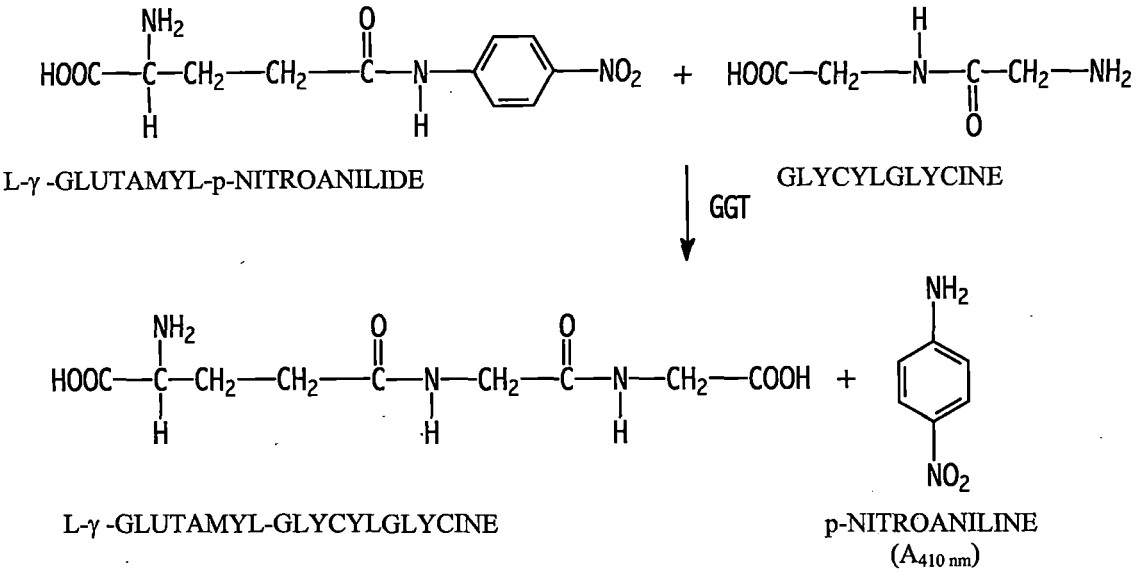
2.6 GGT ACTIVITY ASSAY

GGT activity was assayed according to the method as described by Meister *et al.*[15], with slight modifications.

Assay principle

In presence of the substrate L-γ-glutamyl-p-nitroanilide and glycylglycine as the acceptor, GGT catalyzes the transfer of the L-γ-glutamyl moiety to glycylglycine forming L-γ-glutamyl glycylglycine and releasing p-nitroaniline, which absorbs at 410 nm. The activity of the enzyme can be measured by following the increase in absorbance at 410 nm spectrophotometrically.

Reaction mechanism



Reagents

Buffer: 0.1 M Tris HCl, pH 8.0 at 25°C.

Glycylglycine: 0.1 M, pH 8.0, prepared in H_2O and pH adjusted with 2 N NaOH.

L-γ-glutamyl-p-nitroanilide: 2.5 mM, pH 8.0, prepared by dissolving in 20 ml of 0.5 N HCl followed by addition of 30 ml of H_2O, pH was adjusted with Trizma Base and the final volume was made up to 60 ml with H_2O.

Procedure

The following additions were made to a spectrophotometer quartz cuvette (1 ml): –

920 µl Tris HCl buffer, 20 µl L-γ -glutamyl-p-nitroanilide and 10 µl glycylglycine. The solution mixture was brought to 37°C in a thermostatted cuvette holder of the spectrophotometer. Reaction was initiated by adding 50 µl of the enzyme preparation and the rate of release of p-nitroaniline was recorded at 410 nm (ϵ =8800 $M^{-1}cm^{-1}$). The specific activity was expressed as units per mg protein. Protein concentration was determined by Bradford's method of protein estimation, using BSA as standard.

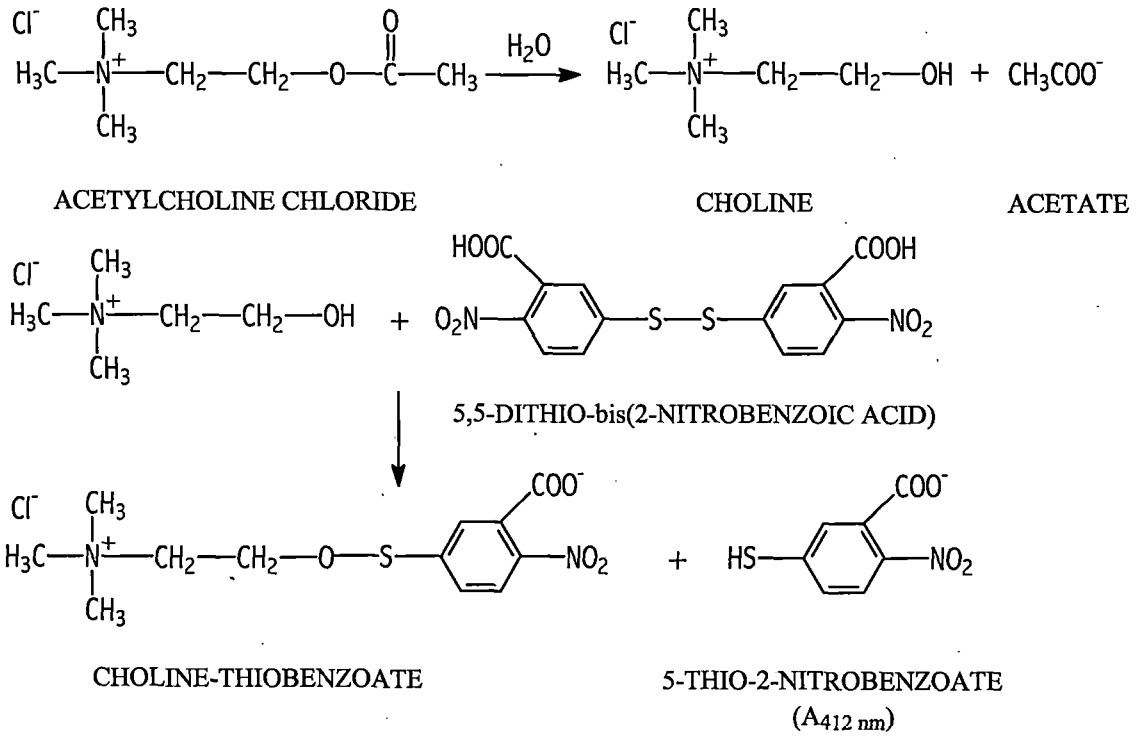
2.7 AChE ACTIVITY ASSAY

AChE activity was assayed according to the methods of Ellman *et al.*[42], and Plummer & Wright[170] with slight modifications.

Assay principle

Acetylcholine chloride, the ester of choline and acetic acid, was used as the substrate in this assay system. AChE hydrolyses acetylcholine chloride to choline and acetate. Choline reacts with an oxidizing agent, 5,5'- dithiobis-(2-nitrobenzoic acid) (DTNB), which splits into two products, one of which i.e. 5-thio-2-nitrobenzoate, absorbs at 412 nm. The activity of the enzyme can thus be measured by following the increase in absorbance at 412 nm spectrophotometrically.

Reaction mechanism



Reagents

Buffer: Sodium phosphate 0.1 M, pH 8.0

DTNB: 10 mM, freshly prepared in buffer containing $NaHCO_3$.

Acetylcholine chloride: 158.5 mM, freshly prepared in buffer.

Procedure

The following additions were made to a spectrophotometer quartz cuvette: –

50 µl of the enzyme preparation was added to 3.0 ml of sodium phosphate buffer and incubated at room temperature for 5 min. Then, 10 µl of DTNB was added and the reaction was initiated by the addition of 20 µl of acetylcholine chloride. The rate of release of 5-thio-2-nitrobenzoate was recorded at 412 nm ($\in$=1.36x10^4 litres $mol^{-1}cm^{-1}$). The specific activity was expressed as units per mg protein. Protein concentration was determined by Bradford's method of protein estimation, using BSA as standard.

2.8 PROTEIN ESTIMATION

Total protein content of enzyme preparations was determined by the method of Bradford[26] (1976), using BSA as standard.

Reagents

Reagent A – Coomassie Brilliant Blue G-250 (0.2 g in 100 ml of 95% ethanol).

Reagent B – Phosphoric acid, 85%.

Procedure

Stock solution was prepared by mixing 50 ml of Reagent A and 100 ml of Reagent B, this was thoroughly mixed and stored in a dark bottle at 4°C. The working solution was prepared by diluting 15 ml of the stock solution to 100 ml, with distilled water, and this was filtered through Whatman filter Paper No. 1 and the resultant filtrate was used immediately for the estimation. The final concentration of the reagents was 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% ethanol (v/v) and 8.5% phosphoric acid (v/v). A diluted protein sample (0.1 ml) was added to 5 ml of the working solution, thoroughly mixed in a cyclomixer, followed by incubation at room temperature (25°C) for 10 min. The intensity of colour developed was measured at 595 nm. Protein concentrations were determined using a computed standard curve prepared by different concentrations of BSA (0.01 – 0.1 mg/ml).

2.9 DETERMINATION OF TOTAL CELL COUNTS AND VIABLE CELL NUMBER

In both the DNA and RNA synthesis assays, the procedures were initiated with a particular number of viable cells. The cell viability test was performed by Trypan Blue dye exclusion method, using a Neubauer hemocytometer as described in Sigma bulletin. This is based on the principle that cells when exposed to Trypan Blue dye, viable cells do not take up the dye, whereas non-viable cells take up the dye and stain blue.

Reagents

A 0.4% Trypan Blue solution was prepared in 0.81% sodium chloride and 0.06% di basic potassium phosphate, and was sterile filtered.

Procedure

Freshly excised liver was rinsed in chilled normal saline (0.9% NaCl), blotted dry and cut into tiny bits of pieces in 1 ml of chilled DMEM containing collagenase. This was left for about 2 min. in ice. It was next subjected to gentle and slight homogenization in ice. The resulting cell suspension was then ready for counting. Trypan Blue solution (0.5 ml) was transferred to an appropriate volume of the cell suspension and the volume was made up to 1 ml with chilled DMEM. This was then incubated for 10 min. at 37°C after which, an appropriate volume of the cell suspension was transferred onto a Neubauer hemacytometer and the cells were counted under a phase contrast microscope. The whole process was carried out as quickly as possible.

The calculations were carried out using the following formulae: -

Cells per ml = the average count per square × dilution factor $\times 10^4$ (count 10 squares).

Total cells = cells per ml × the original volume of fluid from which cell sample was removed.

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100

2.10 RNA SYNTHESIS ASSAY BY URIDINE-5-T TRITIUM ([^{3}H]-U) INCORPORATION

Assay principle

When [^{3}H]-U is added to a cell culture, metabolically active cells will incorporate the labeled precursor into the RNA. The amount of the labeled precursor incorporated is then quantitated by LSC.

Reagents

DMEM, 15% TCA, 5% TCA and [^{3}H]-U.

Dilution of [^{3}H]-U was done with 50% ethanol.

Procedure

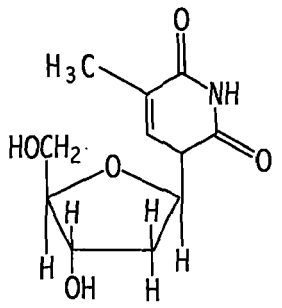
Viable hepatocytes (3×10^6) were taken in 2.0 ml DMEM and incubated for 5 min. at 37°C. Then, 5 μCi of [^{3}H]-U was added to the cell culture and re-incubated for 30 min. at 37°C. During this labeling period, [^{3}H]-U got incorporated into RNA of proliferating cells. The reaction was arrested by the addition of 2.0 ml of chilled 15% TCA, followed by thorough mixing and incubation on ice for 15 min. Cells were harvested by vacuum aspiration onto (15% TCA) pre-wetted glass microfibre filters (GF/C; 25 mm). While free [^{3}H]-U was washed through the filters, the incorporated [^{3}H]-U in the RNA was retained. The harvested cells on the filter discs were washed with 5% TCA (5 ml x 3) and the filters were air-dried. The amount of radioactivity retained on the filters was quantitated by liquid scintillation counter, following addition of 10 ml of the scintillation fluid (Cocktail 'T').

2.11 MEASUREMENT OF CELL PROLIFERATION USING 5-BROMO-2'-DEOXY-URIDINE, (BrdU) LABELING KIT

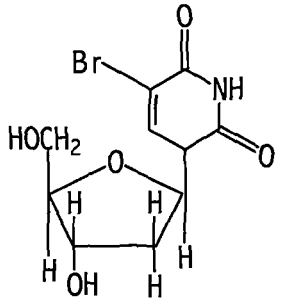
This assay was performed as described initially by Gratzner[58] and by subsequent workers[159, 57, 106, 107]. BrdU an analogue of thymidine, was used for this assay.

Molecular structure of thymidine and BrdU

Thymidine
(5-methyluracil-2'-deoxyribose)



5-Bromo-2'-deoxyribose
(5-Bromouracil-2'-deoxyribose)



Assay principle

The assay is a cellular immunoassay, which uses a mouse monoclonal antibody directed against BrdU based on the cell ELISA principle. Cells cultured in a microtiter plate are incubated with BrdU.The cells are fixed with 0.5 M ethanol/HCl, and are then incubated with nucleases to partially digest the DNA. Incorporated BrdU is detected with the monoclonal anti-BrdU-POD, Fab fragments, and the bound conjugate is visualized with the soluble chromogenic substrate ABTS and measured spectrophotometrically at 405 nm using an ELISA reader.

Reagents

5-Bromo-2'-deoxy-Uridine Labeling and Detection Kit III (Boehringer Mannheim) was commercially obtained. The supplied kit contained the following: -

1. BrdU labeling reagent, (1000x). 1 ml containing 10mM BrdU stock solution (1000x), in phosphate buffered saline (PBS), pH 7.4; sterile.
2. Washing buffer concentrate (10x) , 125 ml PBS, 10x concentrated.
3. Incubation buffer, 125 ml containing 66 mM Tris buffer, 0.66 mM $MgCl_2$, 1 mM 2-mercaptoethanol.
4. Nucleases, stabilized and lyophilized.
5. Anti-BrdU-POD, Fab fragments, one glass vial containing anti-BrdU (monoclonal antibody, Fab fragments from mouse) conjugated with peroxidase (25 U), stabilized, lyophilized.

6. Substrate buffer, 125 ml ABTS-substrate buffer (sodium perborate and citric acid/phosphate buffer).

7. ABTS-substrate, 2,2'-azino-di-[3-ethylbenzthiazolin-sulfonat (6)]. One glass vial containing ABTS-substrate powder for 125 ml substrate buffer.

8. Substrate enhancer, one glass vial containing 125 mg substrate enhancer.

Preparation of working solutions

Solution I. BrdU labeling solution. The vial of the BrdU labeling reagent was diluted with sterile culture medium in the proportion of 1:90, (resulting concentration: 110 µM BrdU). Freshly prepared.

Solution II. Washing buffer. Washing buffer concentrate (10x) was diluted with redist.water in the proportion of 1:10.This washing buffer (solution II), was used to (a) prepare the anti-BrdU-POD, working solution and (b) wash cells after incubation with anti-BrdU-POD.

For other washing steps culture medium containing 10% FCS (fetal calf serum) was used.

Solution III. Incubation buffer. Ready to use, and it is used for diluting the nucleases.

Solution IV. Nucleases. Stock solution was diluted with incubation buffer in the proportion of 1:100.

Solution V. Anti-BrdU-POD, Fab fragments. The whole content of the supplied vial was dissolved in 1.25 ml redist. water (final concentration: 20 U/ml).

Solution Va. Anti-BrdU-POD, Fab fragments. Freshly prepared by diluting the supplied stock solution with washing buffer (Solution II) in the proportion of 1:100, supplemented with 10 mg/ml BSA.

Solution VI. Peroxidase substrate. A vial of supplied ABTS powder was dissolved in substrate buffer, stirred at room temperature till a clear solution was obtained.

Other reagents

1. *Culture medium:* DMEM was sterilized by autoclaving and the following were added: -10% heat inactivated FCS, 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine -monohydrate and streptomycin.

2. *Fixative*: Ethanol p.a. (70%) in HCl (final concentration 0.5 M) i.e. for one microtiter plate, 7 ml ethanol p.a., 100%, was diluted with 2.33 ml redist. water and 670 µl of HCl p.a., 25% was added. This was stored at -20°C and used pre-cooled.

3. *Washing medium:* Culture medium containing heat inactivated 10% FCS.

Procedure

The assay was initiated with 3×10^4 viable hepatocytes taken in 100 µl of the cell culture medium in 96-well microtiter plates. The cell suspension was labeled with 10 µl of freshly prepared working BrdU labeling solution, added to each well and incubated for 6 h at 37°C. During this incubation period, BrdU got incorporated into freshly synthesized DNA. The cell suspension was centrifuged at 300 × *g*, using a rotor device for centrifugation of MTP, for 10 min. at room temperature. The labeling medium was carefully discarded and the adherent cells were washed twice with the wash medium (250 µl × 2). The cell suspension was air dried in an incubator at 60°C for 2 h. The air dried cell suspension was next fixed with 250 µl of the pre-cooled (-20°C) fixative and incubated for 30 min. at –20°C. The fixative was discarded and the wells were washed with wash medium (250 µl × 3). The cells were then incubated with 100 µl of nucleases per well at 37°C for 30 min. to partially digest the cellular DNA and thus, exposing the incorporated BrdU to immunodetection. The nucleases were discarded and washed with wash medium (250 µl × 3), the wash medium of the last wash was carefully removed and 100 µl of peroxidase-conjugated Anti-BrdU antibody (Anti-BrdU-POD) was added to each well and incubated for 30 min. at 37°C. Anti-BrdU-POD located the BrdU label in the DNA and bound to it. The unbound antibody conjugate was removed and the cells were washed with washing buffer (Soln. II) (250 µl × 3). The washing buffer of the last wash was removed carefully and 100 µl of the peroxidase substrate, 2,2'-azino-di-[3- ethylbenzthiazolin-sulfonat (6)] (ABTS) was added to each well. The peroxidase enzyme catalyzed the cleavage of the substrate yielding a green coloured reaction product, which was clearly distinguishable from the colour of the pure peroxidase substrate. Absorbance was read using an ELISA reader at 405 nm. The absorbance of the sample determined was directly correlated to the level of BrdU incorporated into cellular DNA. All the washing steps were carried out at room temperature.

2.12 PREPARATION OF HISTOLOGICAL SECTIONS

The histological sections of the liver tissue of the normal and DEN treated animals were prepared following the method as described by Ratcliffe[111]. The procedure involved the following steps: -

Fixation

The fixative used was Bouin's fluid, which had the following composition:

Picric acid (saturated aqueous solution)	75 ml
Formalin (40% aqueous)	25 ml
Acetic acid (glacial)	5 ml

Freshly excised liver tissue was immediately placed in about 6 ml of freshly prepared Bouin's fluid and cut to approximately 0.4 cm and fixed for 24 h in the same fixative. Excess fixative was washed overnight in running tap water.

Dehydration

Dehydration was carried out in a graded series of ethanol, placing the tissue serially in 30%, 50%, 70%, 90% and absolute alcohol; leaving for about 2 h in each grade and, about 3 h in absolute grade with 3 changes.

Clearing

This step involved placing the tissue in xylene for 2-6 h till the tissue became translucent.

Wax infiltration and casting

Wax infiltration impregnates and surrounds the tissue providing support and prevents the collapse and distortion of the tissue during sectioning. Wax infiltration was done by the xylene method: -

(1) From absolute alcohol ——> xylene (1 change)	2 h
(2) 50: 50 xylene: wax ——— in oven (58°C-60°C, 1 change)	1 h
(3) Molten wax ——— in oven (3 changes)	1 h each

The tissue was transferred from the last wax change and embedded in fresh molten wax and allowed to cool gradually. Care was taken not to overheat the casting blocks.

Section cutting (microtomy) and mounting

The embedded tissue was fixed to a specimen holder (wooden cube) which fitted on the microtome, and sectioned with a sharp knife at 7 μm. The sections were handled with a moist paint brush.

Mounting

Glass slides were uniformly smeared with undiluted egg albumen and the sections were mounted on the slides. A drop or two of water was added on each slide, and gently passed over a hot plate in order to flatten the mounted sections. The slides were dried overnight at 37°C.

Staining

Haematoxylin and eosin procedure was used for staining. Haematoxylin stains the nuclei dark blue and eosin stains the cytoplasm pink. This step involved several sub-steps: -

(1) The slide were de-waxed in xylene	5-10 min.
(2) Absolute ethanol	2 min.
(3) 90% ethanol	1 min.
(4) 70% ethanol	1 min.
(5) Haematoxylin	10-15 min.

(6) Excess stain was removed by acid alcohol mixture (100 ml of 70% ethanol, 0.5 ml concentrated HCl) followed by washing in tap water.

(7) The slides were dipped in 70% ethanol for few seconds.

(8) Eosin (70% ethanol)	5-10 min.
(9) 70% ethanol	1 min.
(10) 90% ethanol	10-15 sec.
(11) Absolute ethanol	2-3 min. x 2

(12) Cleared in fresh xylene.

(13) Mounted in 1-2 drops of D.P.X. and covered with cover slips. Care was taken not to allow the slides to dry up at any stage.

Observation

Finally, the prepared slides were observed and studied under a phase contrast microscope, Leitz Dialux 20.

2.13 PERCHLORIC ACID EXTRACTION OF LIVER MEMBRANE GLYCOPROTEINS

Perchloric acid extraction method specifically extracts membrane surface glycoproteins. The method used in this study was as described by Gold[56, 41] with slight modifications.

Reagents

(1) 2 M Perchloric acid, (2) 1 M Perchloric acid, (3) 1 N NaOH, (4) 0.04% Sodium azide.

Procedure

A crude liver extract was prepared by homogenizing 1 g of liver tissue in 1 ml of distilled water for 2 min. Afterwards, 2 ml of 2 M Perchloric acid was added with stirring, and the resulting thick suspension was centrifuged at 27,000 x g for 15 min. at 3°C. The precipitate was re-extracted with 0.75 ml of 1 M Perchloric acid. The extracts were immediately neutralized with 1 N NaOH and the combined solutions were dialyzed exhaustively against water at 3°C and adjusted to 0.04% sodium azide. The dialyzed solution was concentrated 50-fold by air blowing at room temperature over the dialysis tubing. The non-immuno-reactive precipitate was removed by centrifugation and the resulting supernatant was used for electrophoretic studies.

2.14 SDS -PAGE ANALYSIS

Sodium dodecyl sulphate [CH_3 $(CH_2)_{10}$ $CH_2OSO^-_3$ Na^+] abbreviated SDS is a detergent that readily binds to proteins. SDS-PAGE with discontinuous buffer systems is one of the most commonly used electrophoresis methods today, and is widely used for molecular weight measurements and of polypeptides[119, 109, 16]. Discontinuous buffer systems in combination with polyacrylamide gradient gels enhance band sharpness.

SDS- PAGE was carried out as prescribed in the Pharmacia Biotech PhastSystem™ User Manual.

PhastGel electrophoretic media

The PhastGel electrophoretic media supplied are as follows: -

PhastGel gradient. Pre-casted gradient gels having 13 mm stacking gel zone (4,5 % T, 3 % C) and a 32 mm gradient gel zone. Phast Gel gradient 10-15 has a continuous 10 to 15 % gradient gel zone with 2 % cross-linking. The gels were approximately 0.45 mm thick. The buffer system in the gel was of 0.112 M acetate (leading ion) and 0.112 M Tris, pH 6.4. The gels were available bonded to a transparent polyester backing (0.175 mm thick) and the gel surface was covered with a plastic film which should be peeled off before use. These gels were stored refrigerated.

PhastGel buffer strips. Buffer strips available pre-packed in air-tight packages were used. The buffer system was of 3 % high quality agarose, which had been countercharged and therefore had a low electro endosmosis and containing 0.20 M tricine (trialing ion), 0.20 M Tris and 0.55 % SDS (analytical grade) pH 8.1, approximately 2.5 ml.

Sample buffer. The sample buffer used, had the following components: - 10 mM Tris / HCl, 1 mM EDTA; pH 8.0, 2.5 % SDS, 5.0 % β-mercaptoethanol, 0.01 % bromophenol blue.

Stain: PhastGel Blue R. PhastGel Blue R is a Coomassie R 350 stain stamped into convenient tablet form.

Stock solution: - 1 PhastGel Blue R tablet was dissolved in 80 ml of distilled water and stirred for 5-10 minutes. 120 ml of methanol was added and stirred for 2-3 minutes. This gave a 0.2 % solution.

Final solution: - 1 part of the filtered stock solution was thoroughly mixed with 1 part of 20 % acetic acid in distilled water. The final working solution was freshly prepared.

Destain. 150 ml methanol and 50 ml acetic acid was added to 300 ml of distilled water. (methanol: acetic acid: water =3: 1: 6).

Preserving solution. 10 % glycerol and 10 % acetic acid in water.

The stain, destain and preserving solution were stored in separate dark bottles and were connected to separate IN-Ports in the development unit of the instrument.

Molecular weight measurement. Pharmacia LKB HMW and LMW calibration kit proteins were supplied as dried mixtures. The HMW and LMW vials were reconstituted in 200 µl of SDS buffer, composed of 10 mM Tris/HCl, pH 8.0; 1.0 mM EDTA with 2.5 % SDS and 5.0 % β-mercaptoethanol. This was thoroughly mixed and the mixture was heated at 100°C for 5-10 minutes.

Operation

The entire PhastSystem electrophoretic technique comprises of two main phases: -

(1) Separation Step and (2) Development Step, which further consist of sub-steps.

Separation phase

Sample preparation and loading sample applicators

The dialyzed and concentrated perchloric acid mouse liver extract was mixed with sample buffer in equal proportions (1: 1), denatured by heating at 100°C for 5 minutes and then

centrifuged to remove any insoluble material. The reconstituted Pharmacia LKB HMW and LMW calibration kit proteins were treated similarly.

In each well of a sample-well stamp, 2 µl of the extract sample and the calibration proteins were separately taken. The samples were applied to gels with PhastGel sample applicator 8 / 0.5, which would apply eight samples, each approximately 0.5 µl to the gel. The applicator was lowered to the surfaces of the samples, in the wells of a sample- well stamp and drawn up into the applicator capillaries by capillary action.

Positioning the gels in the gel compartment

Refrigerated SDS- PAGE (10-15 %) PhastGel was carefully removed using the plastic tab of the gel backing as the handle and placed in the gel compartment containing a drop or two of water. It was positioned so that it was in perfect alignment with the outline of the marked gel area, (avoiding any formation of air bubbles) with the gel surface facing upward. The protective plastic film was gently peeled off from the gel surface.

Refrigerated PhastGel SDS buffer strips were inserted into the compartments of the lowered (onto the separation bed) buffer strip-holder; one in the anode and one in the cathode compartment. These were then gently pressed down to ensure good contact between the buffer strips and the gel. The electrode assembly was lowered so that the outer electrodes (the cathode and the anode farthest from it) rest evenly on the buffer strips. The electrodes must have complete and even contact with the buffer strips and this was ensured by gently pressing down the top of the electrodes.

Sample application

The loaded sample applicator arm was lowered onto the appropriate slot and the separation compartment cover was closed.

The operation of the separation step was performed as programmed into the separation method file in the instrument. The separation method programmed was as follows: -

Sample application down at			1.1	1 Vh		
Sample application up at			1.1	10 Vh		
Separation	1.1	250 V	10.0 mA	3.0 W	15°C	60 Vh
Separation	1.2	50 V	0.1 mA	0.5 W	15°C	0 Vh

The separation phase involved two separation steps, the first step was for electrophoresis; samples were applied to the gel, and the protein components separated according to their

sizes. The second step (optional) was programmed to reduce the risk of proteins migrating off the gel, should the stop alarm be missed.

Development phase

The gel was removed from the separation bed and slided into the lower position of the gel holder, with the gel surface facing upwards. The following developmental method was programmed into the development method file: -

Step no.	Solution	IN- Port	OUT- Port	Time	Temperature
1	Stain	4	0	8 min	50°C
2	Wash / Destain	2	0	5 min	50°C
3	Wash / Destain	2	0	8 min	50°C
4	Wash / Destain	2	0	10 min	50°C
5	Preserving solution	9	0	5 min	50°C

After the development procedure, the gel was immediately dried and preserved.

The Rf values were calculated for the calibration proteins as well as for the extract sample using the formula: –

$$Rf = \frac{\text{Distance traversed by Protein}}{\text{Distance traversed by Bromophenol Blue}}$$

A plot of Rf values of the calibration proteins against log of their respective molecular weights was made, and the molecular weight of the extract sample could be obtained by interpolating from the calibration plot.

2.15 LIPOSOME PREPARATION AND ENTRAPMENT OF AK-2123 INTO LIPOSOMES

The method of liposome preparation chosen was the reverse-phase-evaporation (REV) method with slight modifications as described by Alam *et al.*[17]. This method is reported to yield large unilamellar and oligolamellar liposomes, having a percent entrapment efficiency of about 80%. Three different combinations of lipids and phospholipids i.e. DPPC: Chol: DCP, DMPC: Chol: DCP, Egg: Chol: DCP, all in the molar ratio of 1.0: 0.9: 0.25 (Table 2), were tried out by this method and the lipid/AK-2123 ratio was determined for each combination. Since there was no significant difference in the

lipid/ AK-2123 ratio of all the three lipid combinations, the first combination was chosen for the present study, depending on the convenient availability of the required chemicals.

Table 2 Lipid composition and Lipid/AK-2123 ratio used in Liposome preparation.

Lipid composition (mg)	Molar ratio of Lipids	AK-2123 (μg)	Lipid/AK-2123 ratio
DPPC: Chol: DPC (5.1: 2.5: 1.0)	1.0: 0.9: 0.25	1000	8.6
DMPC: Chol: DCP (5.1: 2.5: 1.0)	1.0: 0.9: 0.25	1000	8.5
Egg PC: Chol: DCP (4.4: 2.5: 1.0)	1.0: 0.9: 0.25	1000	8.0

Procedure

A thoroughly blended mixture of DPPC dissolved in $CHCl_3$, Chol. dissolved in $CHCl_3$, and DCP dissolved in $CHCl_3$: CH_3OH (2: 1) mixture, having the following molar ratio: DCCP: Chol: DCP = 1.0: 0.9: 0.25 was made. The organic solvents were evaporated by flushing nitrogen gas ($N_2\uparrow$) through the lipid mixture, and alternately dipping the test tube in a water bath, whose temperature was maintained at 30°C - 35°C, till a fine film was formed around the inner walls of the test tube. The lipid film was solubilized in minimum amount of $CHCl_3$ (250 μl) followed by vortexing. While vortexing, AK-2123 prepared in normal saline at a concentration of 20 mg/ml, was added drop-wise to the lipid dispersion, taking care to minimize the chloroform: saline emulsion formation. With continuous vortexing, $N_2\uparrow$ was flushed till whatever emulsion formed was rid off. The mixture was centrifuged at 20,000 x *g* for 30 min. at 4°C. The supernatant was preserved; while the pellet was resuspended in normal saline and re-centrifuged as before. The resultant supernatant pooled with that obtained from the first centrifugation contained free AK-2123, and this was used for determining the percent entrapment efficiency of the liposomes by LASER Raman spectroscopy. While the pellet containing liposome encapsulated AK-2123 resuspended in normal saline, was used for the experimental purposes.

2.16 DETERMINATION OF % ENTRAPMENT EFFICIENCY OF LIPOSOMES BY LASER RAMAN SPECTROSCOPY

The principle of this experiment lies in the presence of symmetric stretch of bonds on $-NO_2$ group of AK-2123. At a known wave number of 1313 cm^{-1} for these bonds in Raman spectroscopy, the intensity (I band) of $-NO_2$ group of AK-2123 was measured. To avoid any error due to sample to sample variation or instrumental errors, an internal standard of $(NH_4)_2\ SO_4$ was used because it also exhibits symmetric mode on $-SO_4^{2-}$ group with 986 cm^{-1} wave number[134].

Procedure

A stock solution of AK-2123 (20 mg/ml) was made in normal saline. It must be ensured that the supernatant containing free AK-2123, obtained from the liposome preparation and AK-2123 entrapment step, must be absolutely clear and this was achieved by filtering the supernatant through a 0.2 μ pore size glass microfibre filter. Different standard concentrations of the stock AK-2123 solution i.e. 2.5 mg/ml, 5.0 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml, and 1 ml of the filtered supernatant containing free AK-2123, were taken in different test tubes. The volume was made up to 1 ml with normal saline followed by the addition of 100 μl of $(NH_4)_2SO_4$ as an internal standard, and the spectrum was taken in 1403 Double Monochromator, Spex.

2.17 IRRADIATION

Animals were whole body Gamma (γ) irradiated in a Gamma Chamber 900 (BARC, India) with ^{60}Co as the source of radiation, at a dose rate of 8.4 Gy/min.

2.18 AK-2123 ADMINISTRATION

Stock AK-2123 solution of 5-wt % was prepared in normal saline. AK-2123, both in it's free and liposome encapsulated forms, were administered by intraperitoneal injection at a dose of 200 mg/kg body weight, 30 min. prior to irradiation. Within an hour following irradiation, the animals were killed by cervical dislocation and the liver was used for different assays as described before.

2.19 HAEMATOLOGICAL ANALYSIS

The methods used for the analysis of the haematological parameters namely- haemoglobin, WBC, RBC and whole blood proteins were as described by Dacie and Lewis[74].

Procedure

Blood was drawn by retro-orbital bleeding, and was immediately used for different tests.

2.19.1 Spectrophotometric Determination Of Haemoglobin (Hb) Content In Blood

Reagents

A solution of Potassium ferricyanide, 200 mg and Potassium cyanide, 50 mg was prepared in 1 litre water and pH was adjusted to 9.6.

Whole blood was diluted suitably (1 to 200 or 1 to 250) with the cyanide-ferricyanide reagent. It was stood for 10 minutes and absorbance was read at 540 nm. The Hb content was calculated as follows: -

$$\text{Concentration (g/dl)} = \frac{A^{540}\ \text{HiCN} \times 64\,500 \times \text{dilution factor}}{44.0 \times d \times 1000 \times 10}, \text{ where}$$

A^{540} HiCN = absorbance of the solution at 540 nm.

64 500 = molecular weight of Hb (derived from 64 458),

Dilution factor = 201 when 20 µl of blood is diluted in 4 ml of reagent,

44.0 = millimolar extinction coefficient,

d = layer thickness in cm,

1000 = conversion factor for mg to g,

10 = conversion factor for g/l to g/dl.

2.19.2 Total RBC Counts By Visual Method

Total counts by Visual methods were carried out for the blood cells using Neubauer hemacytometer.

Reagent

Red cell diluting fluid, Gower's reagent was commercially obtained.

Procedure

A 1:200 dilution of blood was made by adding 20 µl of blood to 4 ml of the Gower's reagent. After proper sealing, it was gently cyclomixed and then the cells were counted using phase contrast microscope. The RBCs did not agglutinate and they maintained their normal disc-like form. Calculations were done according to the formula,

Red-cell count per µl = N x 1/0.02 x 200* (dilution)

= N x 10000

= N x 10^4 x 10^6/l.

where, N = number of cells in 80 small squares, 0.1 mm in depth (0.02 µl in volume)

2.19.3 Total WBC Counts By Visual Method

Reagent

WBC diluting fluid, was commercially obtained.

Procedure

A 1 in 20 dilution of blood was made by adding 20 µl of blood to 0.38 ml of the diluting reagent in a test tube. After proper sealing, the cell suspension was gently cyclomixed for at least 1 minute; the cells were then counted using phase contrast microscope. The WBCs remained intact and their nuclei stained deep violet-black; while the RBCs were lysed. Calculations were done according to the formula,

WBC count per µl = N x 10 x 20 (dilution)

= N x 200

= N x 200 x 10^6/l,

where, N = Number of cells in 0.1 µl.

2.19.4 Whole Blood Protein Estimation

Whole blood protein was estimated by the method of Bradford as described earlier.

3 RESULTS

3.1 CARCINOGENESIS INDUCTION STUDIES

Following are the results of the experiments conducted, and they have been represented in graphical as well as in tabular forms. All statistical calculations were done by student's t-test.

3.1.1 General Observations

Animals, on chronic exposure to DEN showed signs of lethargy and sluggishness. A decrease in the body weight was also observed. Majority of the animals survived the complete induction treatment course. The photographs of liver of age-matched normal and DEN treated mice are represented below (Fig.1).

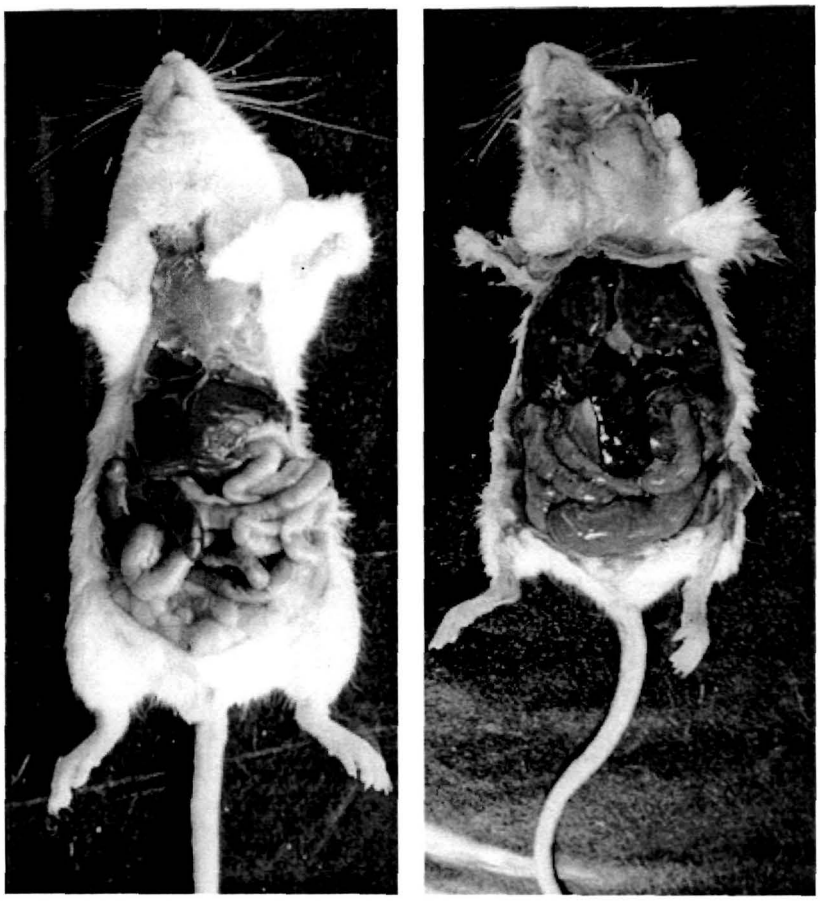


Fig. 1 Photographs showing liver of (**A**) Normal and (**B**) DEN treated mice.

A significant morphological change in the liver of treated mice was observed (Fig.1). The liver was enlarged, it's texture was rough and coarse. In fact, the liver tissue was hardened as compared to it's counterpart that received no DEN treatment. The spleen of

the DEN treated mouse was also affected, it was enlarged and was also somewhat course and hard than that of the normal mouse.

3.1.2 Effects Of DEN On Marker Enzymes

Animals after having received the complete DEN treatment protocol showed marked alterations in their marker enzyme activities (Fig. 2). Control group of animals showed an average GGT activity of 0.003069 U/mg protein while the DEN treated group showed an activity of 0.035551 U/mg protein, thereby exhibiting an elevation of 11.58 folds (Fig. 2A). On the other hand, AChE activity was elevated by 5.485 folds, with the control group of animals showing AChE activity of 0.007584 U/mg protein while the treated group gave an activity of 0.046031 U/mg (Fig. 2B). The differences in the activities of GGT and AChE were statistically highly significant ($P < 0.0001$) against normal (see Table 6 & 7).

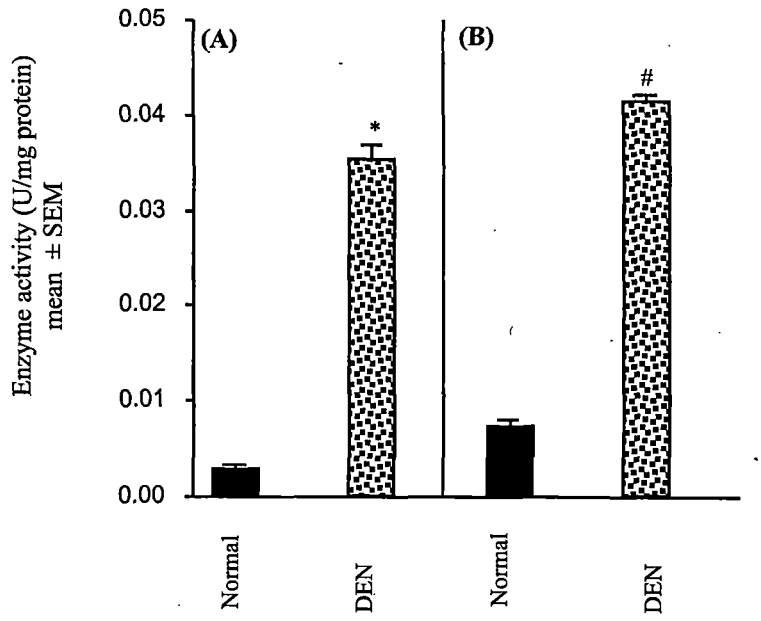


Fig. 2 (A) Liver GGT activity and **(B)** Liver AChE activity in the supernatant fractions of normal mice and those exposed to DEN.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against normal.

3.1.3 Effects Of DEN On The Rate Of Nucleic Acids Syntheses

Results obtained from the DNA and RNA syntheses measurement by BrdU labeling and by [^{3}H]-U incorporation experiments respectively, showed an elevation of 7.12 folds in the rate of DNA synthesis (Fig. 3A), and an elevation of 5.07 times in the RNA rate of synthesis (Fig. 3B). The differences in the synthetic indices of both DNA and RNA when analysed statistically were found to be highly significant ($P < 0.0001$) as against normal (see Table 8 & 9).

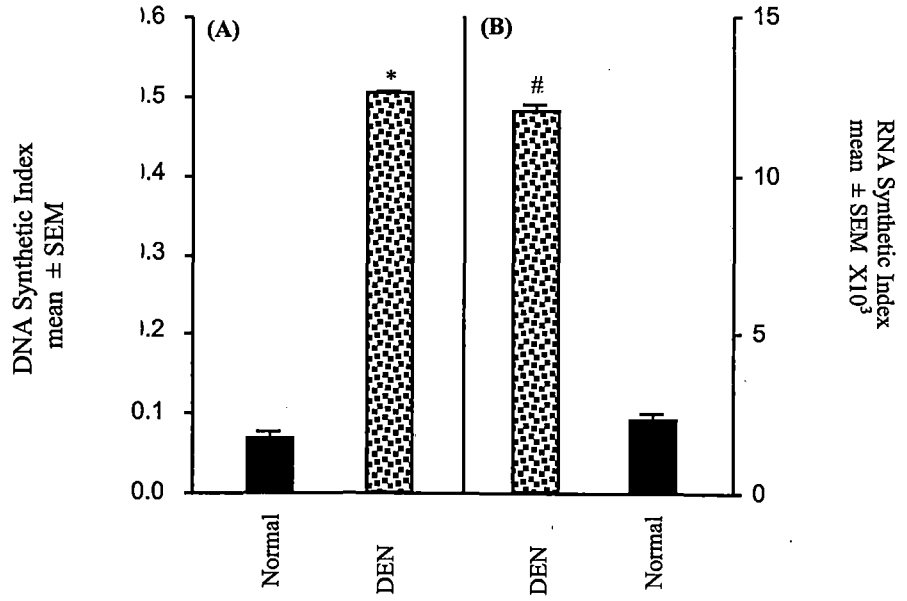


Fig. 3 (**A**) DNA synthetic index and (**B**) RNA synthetic index of hepatocytes of normal and DEN exposed mice.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against normal.

3.1.4 Histological Studies

The histological sections of the liver of DEN treated animals showed a lot of differences and alterations in comparison to that of the normal. The treated liver section showed the following characteristics: (a) loss of regular arrangement of cells, (b) variation in cell shape and size, (c) high increase in the cell number, (d) increased density staining and (e) presence of multinucleated cells; while the normal liver section had well defined, symmetrical, mono and bi-nucleated cells (Fig. 4).

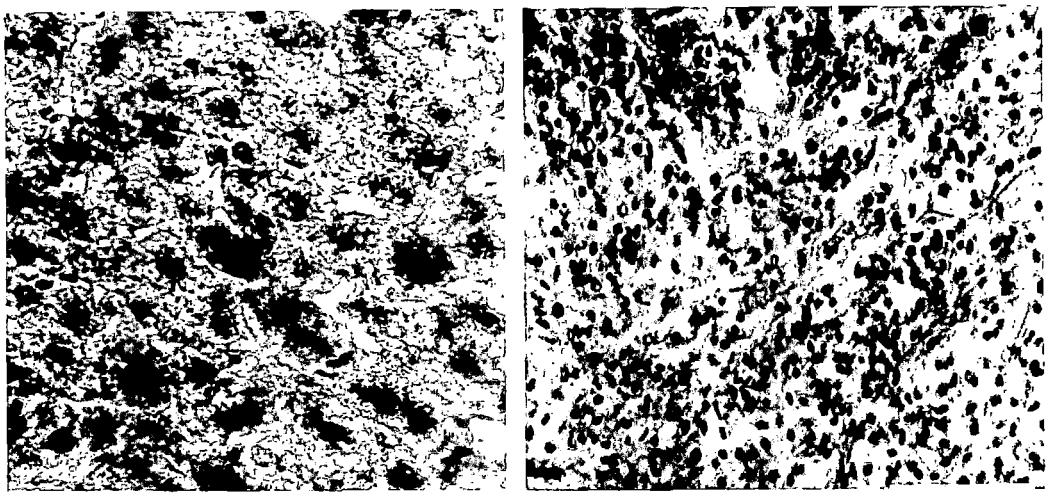


Fig. 4 Liver microphotographs of (**A**) normal and (**B**) DEN treated mice. M X 40.

3.1.5 SDS-PAGE Gel Electrophoretic Studies

The SDS-PAGE Gel of the perchloric acid liver extract of the normal and DEN-treated mice showed no difference in protein profiles. There was, however, a significant

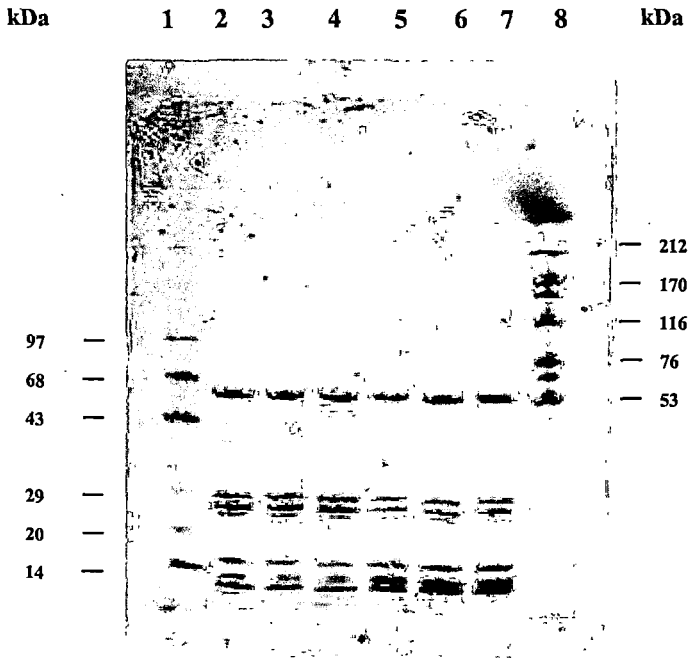


Fig. 5 Perchloric acid extract of liver proteins of normal and DEN treated mice resolved by SDS-PAGE with PhastGel gradient 10-15 and stained with Coomassie Brilliant Blue. The samples from left to right: Lane 1-LMW markers; Lanes 2, 3, 4 - DEN treated; Lanes 5, 6,7 –Normal; Lane 8 – HMW markers.

differential expression of several protein bands, such as those between 20 kDa and 29 kDa, which were over expressed in the treated group; while those between 14 kDa and 20 kDa were under expressed (Fig. 5).

3.2 LIPOSOMES PREPARATION

Liposomes were prepared by the reverse phase evaporation (REV) method as reported earlier (Alam *et al.*, 1992). The liposomes were relatively larger in size and most of them were unilamellar as observed under a phase contrast microscope (Fig. 6).

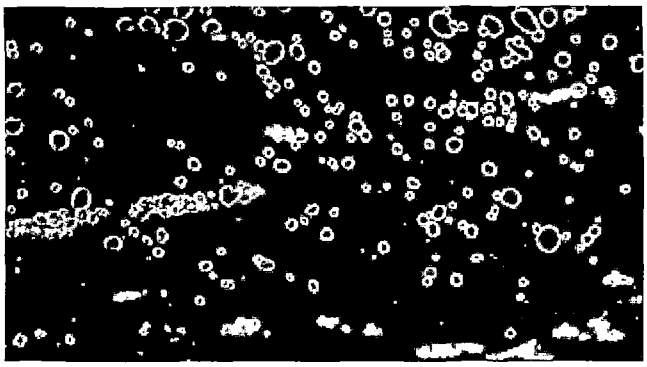


Fig. 6 Microphotograph of liposomes prepared by reverse phase evaporation method. **M** X40.

3.3 PERCENT ENTRAPMENT EFFICIENCY OF LIPOSOMES

Each Laser Raman spectrum of AK-2123 (Fig. 7) depicts a sharp peak at 1313 cm^{-1} for $-NO_2$ group present in AK-2123. The peak area increases proportionately with the increase in the concentration. The standard calibration curve (Fig. 8) was used to determine the concentration of AK-2123 in the unknown sample. AK-2123 entrapped into liposomes was thus calculated by subtracting free AK-2123 from the total concentration of AK-2123.

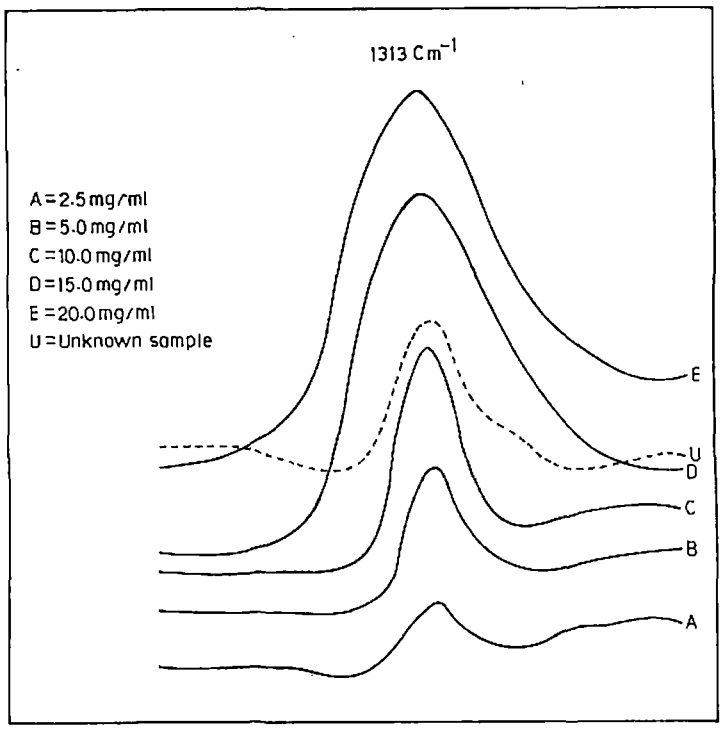


Fig. 7 Spectra of different concentrations of AK-2123 showing different peak intensities. Each spectrum, including that of the unknown concentration of AK-2123, shows a peak at 1313 cm^{-1}.

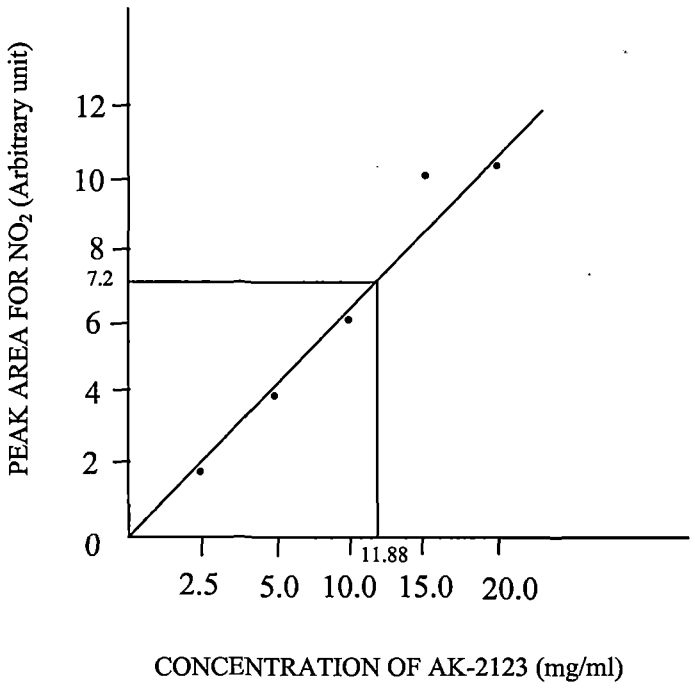


Fig. 8 Standard calibration curve of AK-2123. The concentration of the unknown AK-2123 sample was determined by interpolating from the standard curve.

The concentration of AK-2123 present in each solution was read off from the spectra as follows: -

Peak area = Peak intensity x Full width at half maximum (FWHM)

$$\text{Depolarization } (\rho) = \frac{\text{Peak intensity for perpendicular polarization}}{\text{Peak intensity for parallel polarization}}$$

$$\text{or, } (\rho) = \frac{\text{Peak area for perpendicular polarization}}{\text{Peak area for parallel polarization}}$$

$$\text{Peak position} = \text{Position of ref. line } (cm^{-1}) + \frac{\text{Peak position (in arbitrary unit)}}{\text{Spectrum width (in arbitrary unit)}} \times \text{Spectrum width } (\text{in } cm^{-1})$$

Based on these formulae, the following results were obtained and listed in table 1.

Reference peak intensity = 0.13138 (no. of counts)

Reference peak area = 0.7909

The value of the reference depolarization ratio lies between 0 and ¾

Calculations for AK-2123 at 20 mg/ml: -

Peak intensity for parallel polarization (in counts) = $\frac{80}{220}$ = 0.3636, where 80 = Peak height

Peak intensity for perpendicular polarization (in counts) = $\frac{26}{220}$ = 0.1181, where 26 = Peak height

$$\text{Relative peak intensity} = \frac{I_{peak}^{\nu_1 NO_2}}{I_{peak}^{\nu_1 SO_4}} = 2.76 \text{ for parallel polarization and } 0.90 \text{ for perpendicular polarization}$$

FWHM = 22.9 for parallel polarization and 21.7 for perpendicular polarization.

Peak area ≈ 8.3264 for parallel polarization and 2.5645 for perpendicular polarization.

$$\text{Relative peak area} = \frac{\text{Area } \nu_1 NO_2}{\text{Area } \nu_1 SO_4} = 10.52 \text{ for parallel polarization and } 3.24 \text{ for perpendicular polarization}$$

Peak intensity depolarization ratio (ρ) = 0.3250
Peak area depolarization ratio (ρ) = 0.3076

$$\text{Peak position} = 1275 + \frac{65}{170} \times 100 = 1313.2\ cm^{-1} \text{ for parallel polarization}$$

$$\text{Peak position} = 1275 + \frac{65}{170} \times 100 = 1313.2\ cm^{-1} \text{ for perpendicular polarization}$$

The peak area for each peak was calculated and plotted (Fig. 8) against their respective known concentrations.

Similarly the peak area of the supernatant sample (unknown) was calculated and the value was obtained from the standard curve. it was found to be 11.88 mg/ml.

% free AK-2123 in supernatant = 59.4 %

% of entrapped AK-2123 = 100 - 59.4 = 40.6

Therefore, the % entrapment efficiency of the prepared liposomes = 40.6 %

Table 3 Raman data for ν_1 of NO_2 excited by 488.0 nm of Ar ion Laser at 4 cm^{-1} slit width

AK-2123 (mg/ml)	Peak position (cm^{-1})	Depolarization ratio (Peak intensity)	FWHM (in cm^{-1})	Relative peak area
20	1313.2	0.32	22.9	10.52
15	1313	0.39	22.9	9.97
10	1313	0.35	22.46	6.24
5	1312.3	0.43	22.98	3.91
2.5	1313	0.36	22.5	1.79
Sample (supernatant)	1313	0.42	21.73	7.2

3.4 CARCINOGENESIS REGRESSION STUDIES

3.4.1 % Cell Viability At Different Radiation Doses

This study was carried out by using the Trypan Blue dye exclusion method after the mice were irradiated at different radiation doses. The results of this study as shown in Fig. 9 and in Table 4 indicate that in absence of radiation, the cell viability in all the groups of mice under study was about 95%. With increase of radiation dose, the % viability went down gradually in all the cases. This was the trend till about 4 Gy. At 8 Gy and beyond, although the normal and treated groups followed the expected downward trend, the viability

of DEN treated groups with free and liposome encapsulated AK-2123 plateaued. At 10 Gy and 20 Gy this deviation was very pronounced. Because of this, the entire regression studies were carried out at these two doses of radiation, i.e. at 10 Gy and 20 Gy. The cell viability data are depicted in Table 4.

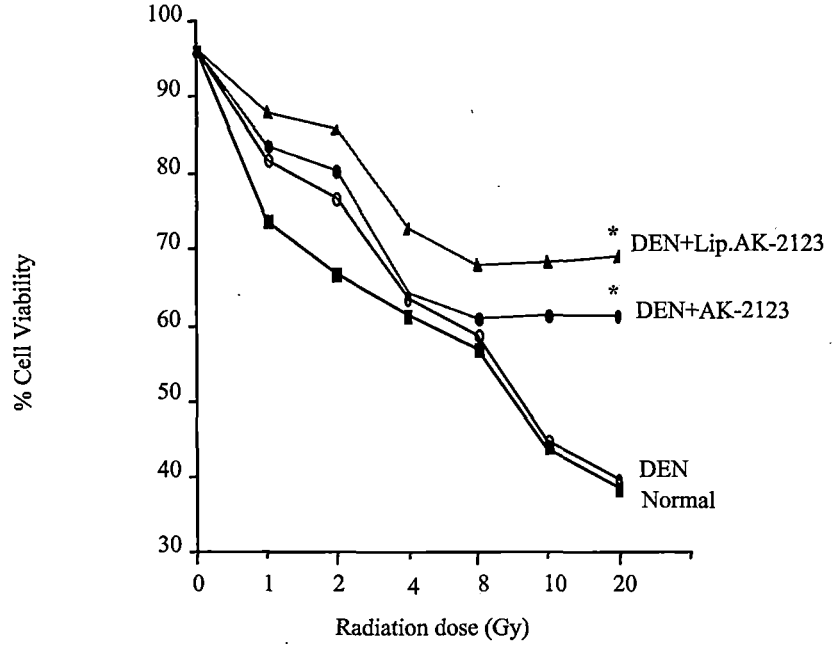


Fig. 9 Percent viability of hepatocytes following whole body γ-irradiation at different doses in normal mice and those exposed to DEN alone, and DEN in presence of free AK-2123 and liposome encapsulated AK-2123. No bar means that the SEM was smaller than the thickness of the point.
* $P < 0.0001$ against DEN treated.

Table 4 % Cell viability at different radiation doses.

Dose (Gy)	Normal	DEN-treated	DEN+Free AK-2123		DEN+Liposomal AK-2123	
	n = 6	n = 6	n = 6		n = 6	
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	*P*	$\bar{x} \pm SD$	*P*
1	73.6±0.615	81.7±0.820	83.5±0.287	<0.0001	87.9±0.230	<0.0001
2	66.8±0.650	76.6±0.604	80.2±0.325	<0.0001	85.4±0.402	<0.0001
4	61.4±0.565	63.4±0.314	64.1±0.318	N.S.	72.5±0.413	<0.0001
8	56.9±0.353	58.6±0.509	60.8±0.255	<0.0001	68.1±0.656	<0.0001
10	43.6±0.381	44.6±0.579	61.1±0.267	<0.0001	68.4±0.470	<0.0001
20	38.4±0.353	39.6±0.763	61.4±0.399	<0.0001	68.0±0.322	<0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

Figure 10 gives the plot of the viability modification factor (VMF) for hepatocytes following whole body gamma irradiation at different doses in DEN-treated mice alone, and in presence of free and liposome encapsulated AK-2123. This factor was calculated to quantify the radioprotective effect of free AK-2123 and liposome encapsulated AK-2123. VMF is a ratio of the % viability of the cells after irradiation in presence of either free, or liposome encapsulated AK-2123 to the % viability of cells after irradiation alone, i.e.

$$\text{VMF} = \frac{\text{\% Viability of cells in presence of free AK-2123 or liposomal AK-2123 after X Gy of } \gamma\text{- radiation}}{\text{\% Viability of cells after X Gy of } \gamma\text{-radiation}}$$

The results obtained shows that at 10 Gy and 20 Gy, DEN treated mice showed a decline in the % viability whereas those with either free AK-2123 or liposomal AK-2123 showed better % viability. DEN treated group in presence of free AK-2123 gave a VMF of 1.3 at 10 Gy and 1.54 at 20 Gy. Whereas, DEN treated group with liposomal AK-2123 gave a VMF of 1.53 at 10 Gy and 1.7 at 20 Gy, indicating that liposome encapsulated AK-2123 shows higher and better VMF than free AK-2123 (see Table 5).

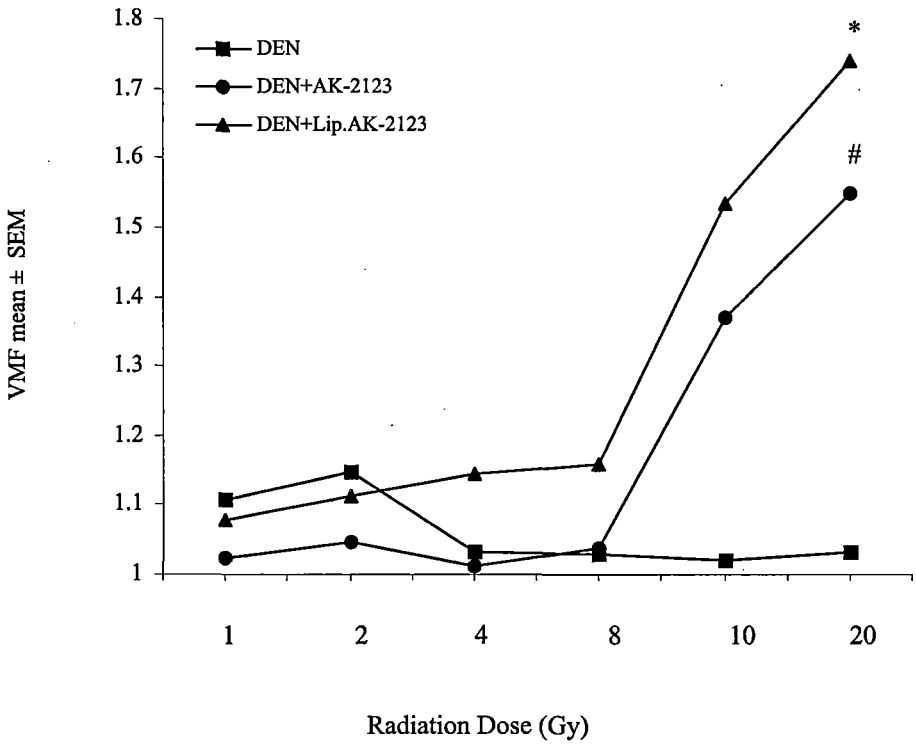


Fig. 10 Viability Modification Factor calculated for hepatocytes following whole body γ-irradiation at different doses in normal mice and those exposed to DEN alone, and DEN in presence of free AK-2123 and liposome encapsulated AK-2123.
# $P < 0.0001$ against DEN treated.
* $P < 0.0001$ against DEN & DEN + AK-2123 + radiation.

Table 5 VMF at different radiation doses.

Radiation dose (Gray)	DEN	DEN+ AK-2123	DEN+Lip.AK-2123
1	1.108	1.022	1.076
2	1.147	1.046	1.113
4	1.032	1.011	1.143
8	1.030	1.037	1.160
10	1.021	1.371	1.534
20	1.031	1.548	1.740

3.4.2 Status Of GGT Following Exposure To Radiation In Presence Of AK-2123

The results of GGT activity on administering free and liposome encapsulated AK-2123 to the DEN treated and age-matched normal mice are shown (Fig. 11 & 12, Table 6).

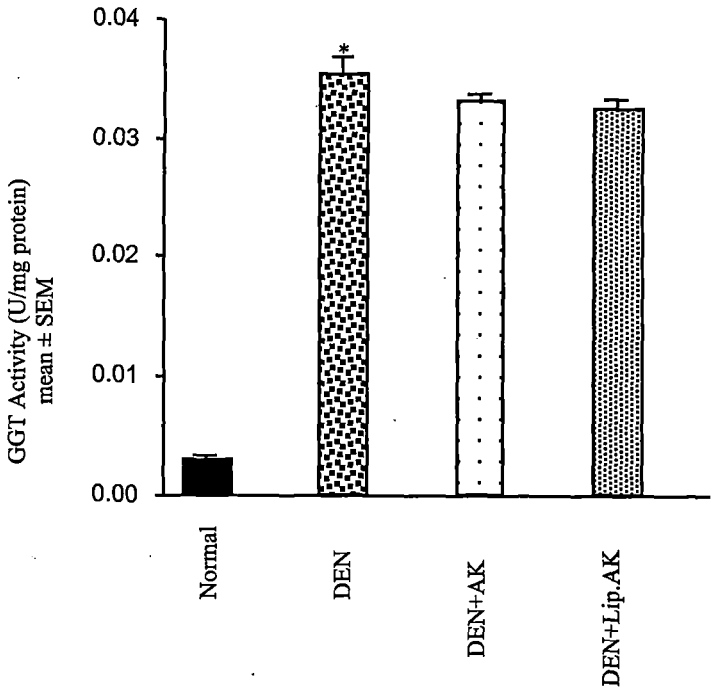


Fig. 11 Liver GGT activity in the supernatant fractions of normal mice and those exposed to DEN, and DEN in the presence of free and liposome encapsulated AK-2123.
*$P < 0.0001$ against Normal.

Fig. 11 shows that as compared to the normal group of mice, the enzyme activity was elevated by about 11 folds following DEN treatment. Administration of free AK-2123 and liposome encapsulated AK-2123 hardly had any significant effect on the GGT activity. Fig. 12 shows that exposure of the DEN treated groups of mice to radiation alone at 10 Gy and 20 Gy resulted in a downfall of the enzyme activity below the normal enzyme level. DEN treated mice exposed to either 10 Gy or 20 Gy in presence of AK-2123 in its free or liposome encapsulated forms showed significant fall in the GGT activity level as compared to that of DEN treated group alone. The fall in the GGT activity level in the group treated with liposome encapsulated AK-2123 was more significant than those treated with free AK-2123, at both doses of radiation. The fall at 20 Gy was even more significant than that at 10 Gy. The data are reproduced in Table 6.

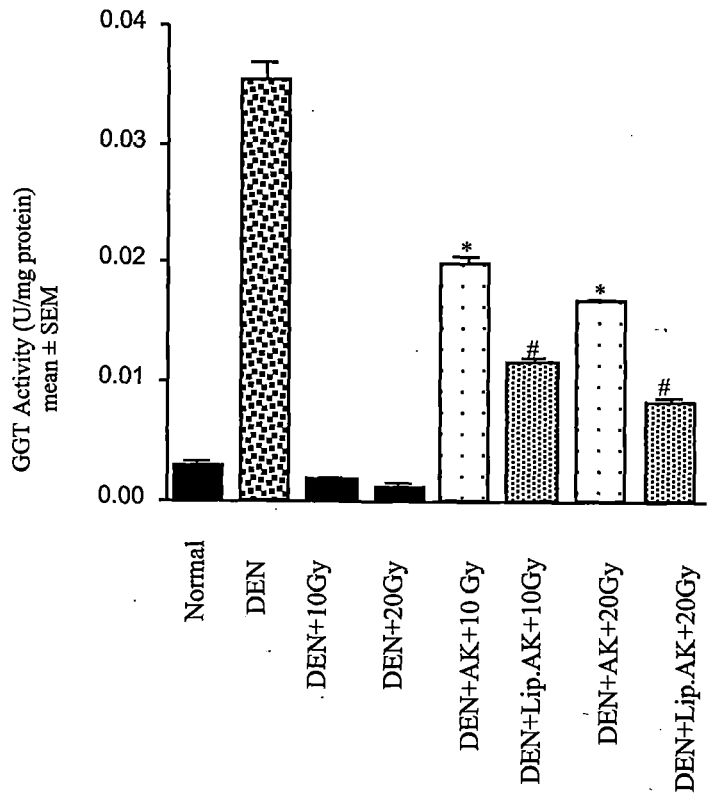


Fig. 12 Liver GGT activity in the supernatant fractions of normal mice and those exposed to DEN followed by irradiation (10 Gy & 20 Gy) in the presence of free and liposome encapsulated AK-2123.
*$P < 0.0001$against DEN, n = 46
#$P < 0.0001$ against DEN & DEN+AK-2123 + radiation.

Table 6 GGT Activity (U/mg protein).

Groups	n	$\bar{x} \pm SD$	*P*
Normal	41	0.003063 ± 0.0013	—
DEN treated	38	0.035551 ± 0.0081	< 0.0001
DEN+Free AK-2123	8	0.033470 ± 0.0008	N.S.
DEN+Liposomal AK-2123	8	0.033001 ± 0.0081	N.S.
DEN+10 Gy.	8	0.002012 ± 0.0003	< 0.0001
DEN+20 Gy.	8	0.001461 ± 0.0002	< 0.0001
DEN+Free AK-2123+10 Gy.	10	0.020248 ± 0.0008	< 0.0001
DEN+Liposomal AK-2123+10 Gy.	10	0.011967 ± 0.0004	< 0.0001
DEN+Free AK-2123+20 Gy.	10	0.017038 ± 0.0004	< 0.0001
DEN+Liposomal AK-2123+20 Gy.	10	0.008487 ± 0.0006	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

3.4.3 Status Of AChE Following Exposure To Radiation In Presence Of AK-2123

The results shown in Fig. 13, 14 and Table 7 are that of AChE marker enzyme.

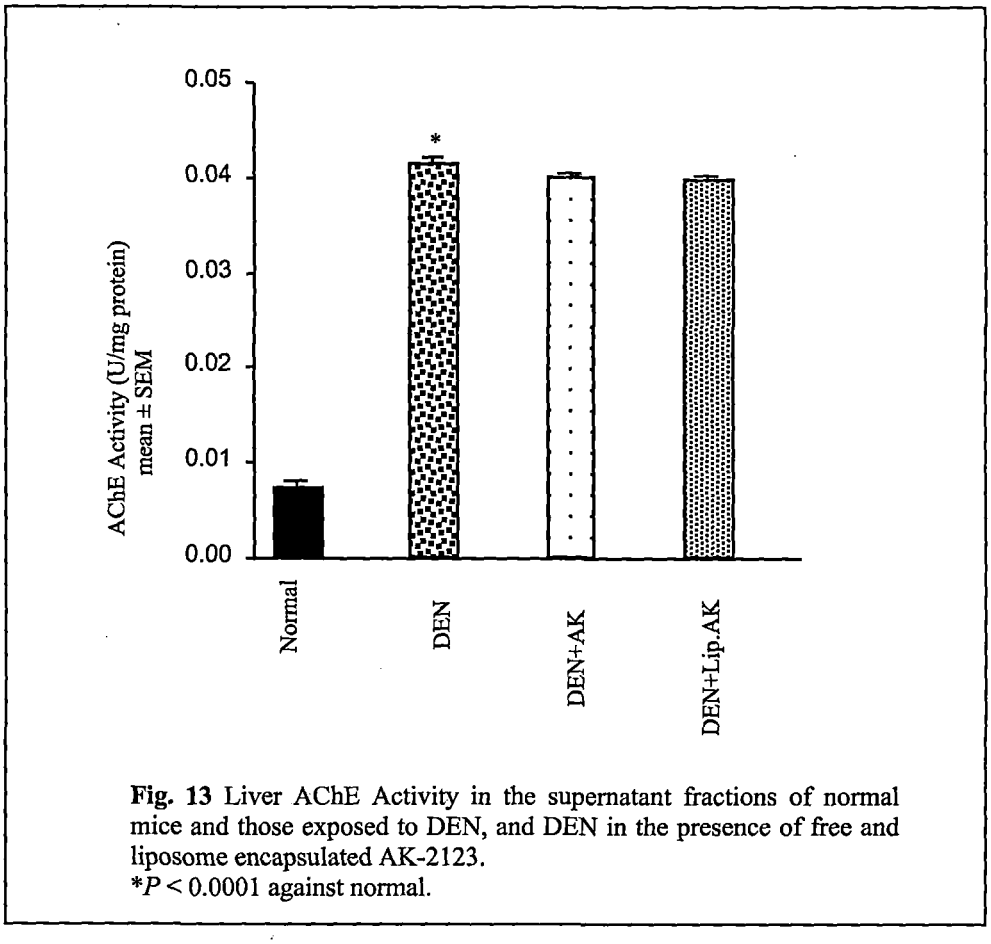


Fig. 13 Liver AChE Activity in the supernatant fractions of normal mice and those exposed to DEN, and DEN in the presence of free and liposome encapsulated AK-2123.
*$P < 0.0001$ against normal.

The AChE activity was enhanced following DEN treatment as compared to the normal group showing more than 5 folds increase (Fig. 13). Administration of free AK-2123 and liposomal AK-2123 did not produce any significant effect on AChE activity level. Fig. 14 shows that irradiation of the DEN treated group of mice at 10 Gy and 20 Gy resulted in a sharp fall of the enzyme activity level, which fell down even below the normal level. The DEN treated mice exposed to 10 Gy or 20 Gy in the presence of AK-2123 in it's free or encapsulated forms, showed a significant decrease in the enzyme level. However, the decrease in AChE activity of DEN treated group, on irradiation at 10 Gy and 20 Gy was more pronounced when AK-2123 was administered after encapsulating into liposomes (Fig. 14).

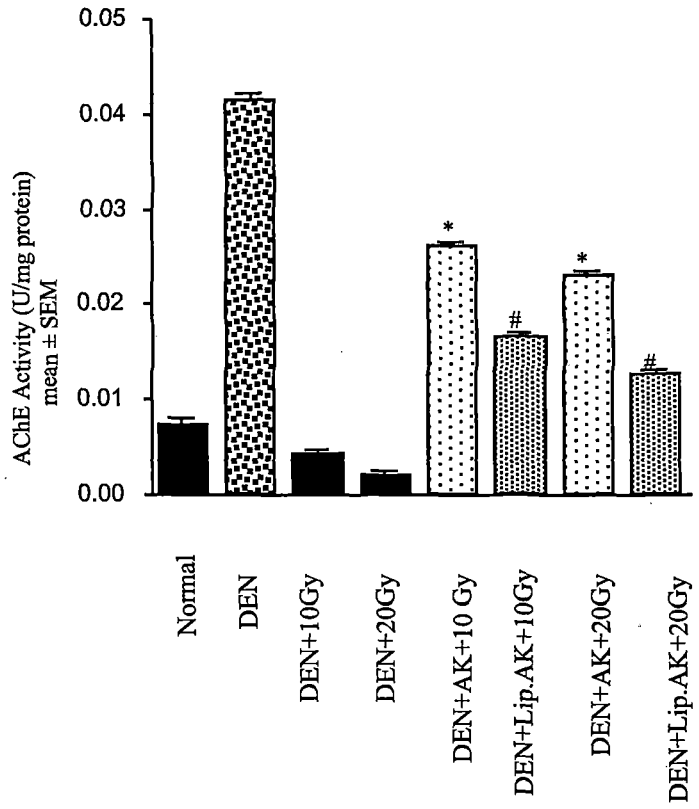


Fig. 14 Liver AChE activity in the supernatant fractions of normal mice and those exposed to DEN followed by irradiation (10 Gy & 20 Gy) in the presence of free and liposome encapsulated AK-2123.
*$P < 0.0001$against DEN.
#$P < 0.0001$ against DEN & DEN + AK-2123 + radiation.

Table 7 AChE Activity (U/mg protein).

Groups	n	$\bar{x} \pm$ SD	*P*
Normal	48	0.007584 ± 0.0032	—
DEN-treated	55	0.041603 ± 0.0060	< 0.0001
DEN+Free AK-2123	8	0.040305 ± 0.0007	N.S.
DEN+Liposomal AK-2123	8	0.039988 ± 0.0004	N.S.
DEN+10 Gy.	8	0.004508 ± 0.0004	< 0.0001
DEN+20 Gy.	8	0.002168 ± 0.0003	< 0.0001
DEN+Free AK-2123+10 Gy.	12	0.026308 ± 0.0006	< 0.0001
DEN+Liposomal AK-2123+10 Gy.	12	0.016952 ± 0.0003	< 0.0001
DEN+Free AK-2123+20 Gy.	12	0.023201 ± 0.0008	< 0.0001
DEN+Liposomal AK-2123+20 Gy.	12	0.013037 ± 0.0004	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

3.4.4 Effects Of Radiation And AK-2123 On DNA Synthesis

The rate of DNA synthesis was carried out by the BrdU labeling method. Results shown (Fig.15), indicate a significant elevation (> 7 folds) in DNA synthesis following

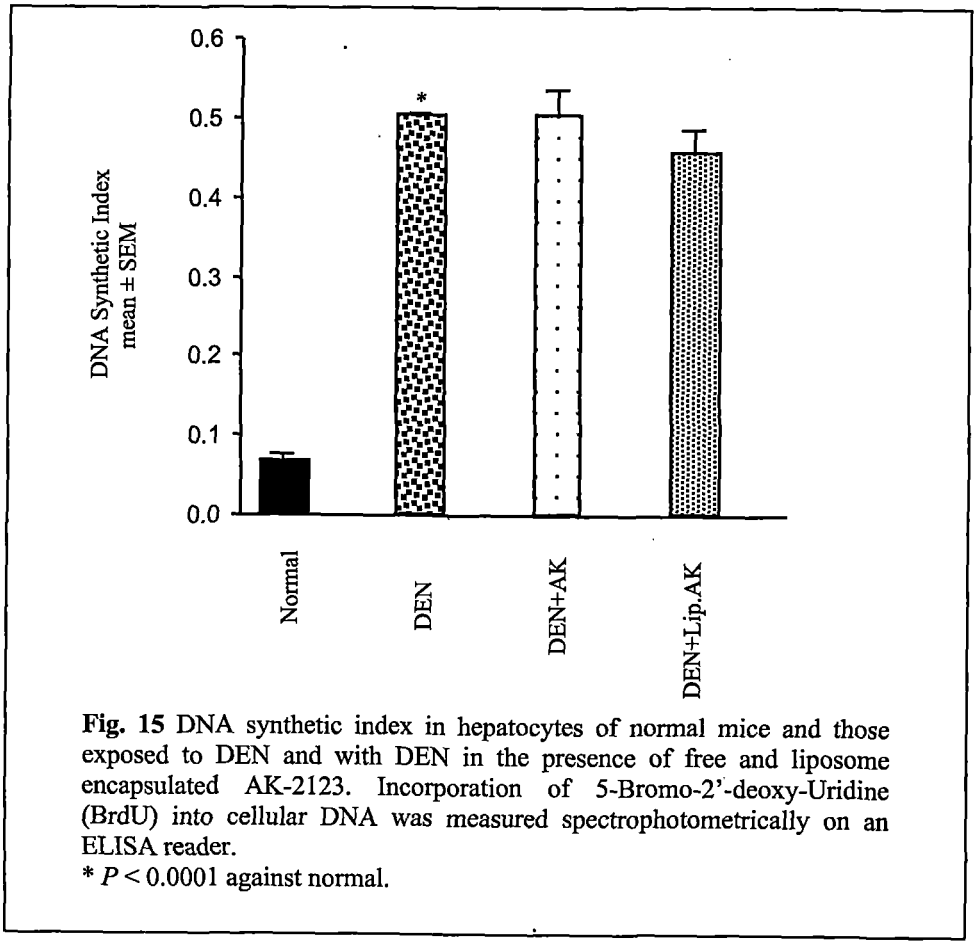


Fig. 15 DNA synthetic index in hepatocytes of normal mice and those exposed to DEN and with DEN in the presence of free and liposome encapsulated AK-2123. Incorporation of 5-Bromo-2'-deoxy-Uridine (BrdU) into cellular DNA was measured spectrophotometrically on an ELISA reader.
* $P < 0.0001$ against normal.

DEN treatment. No effect on the rate of DNA synthesis was observed in the DEN treated mice after administering AK-2123 either in it's free or liposome encapsulated forms (Fig. 15, Table 8). This altered synthetic index was very significantly brought down below the normal level upon exposure to 10 Gy and 20 Gy radiation alone.

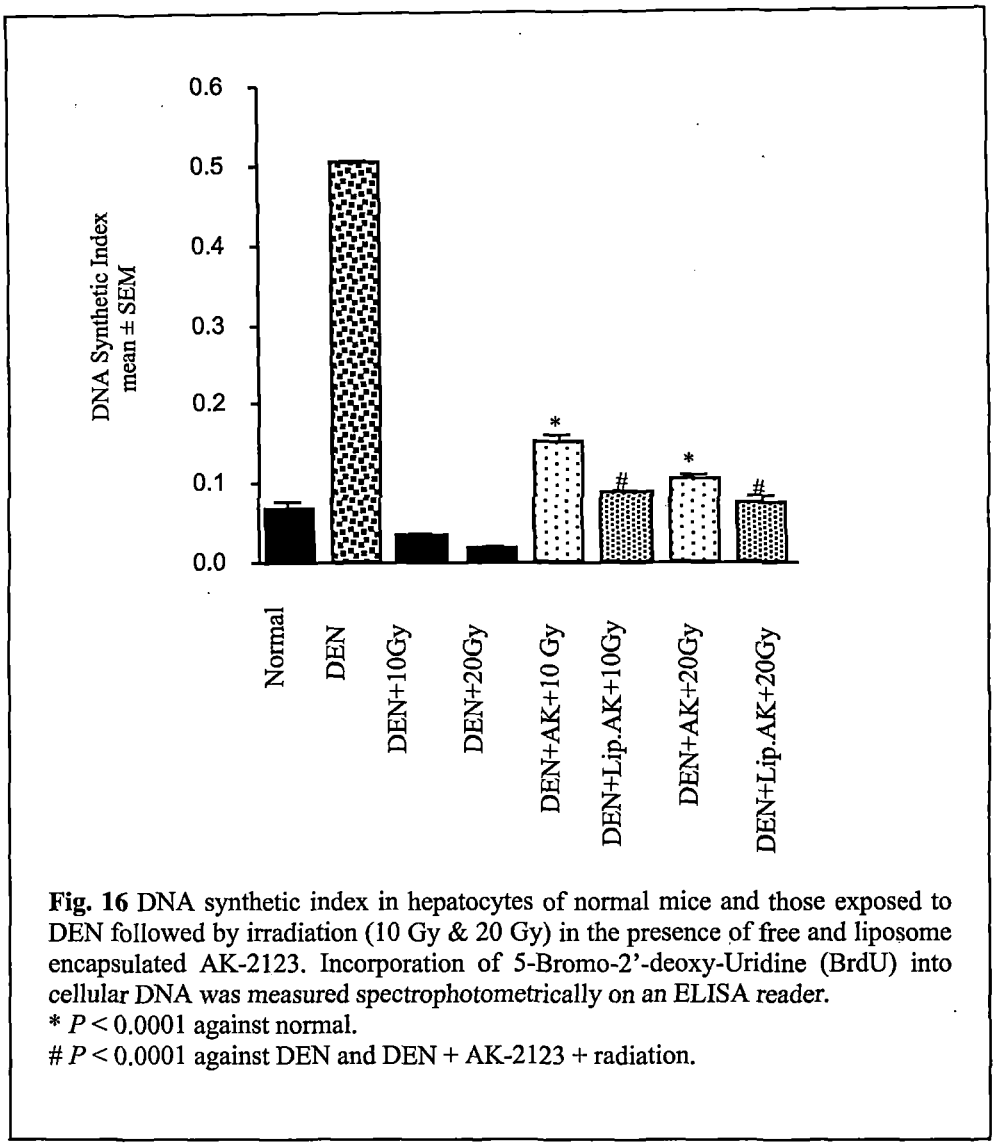


Fig. 16 DNA synthetic index in hepatocytes of normal mice and those exposed to DEN followed by irradiation (10 Gy & 20 Gy) in the presence of free and liposome encapsulated AK-2123. Incorporation of 5-Bromo-2'-deoxy-Uridine (BrdU) into cellular DNA was measured spectrophotometrically on an ELISA reader.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against DEN and DEN + AK-2123 + radiation.

As seen in Fig. 16, the DEN treated group administered with free AK-2123 and irradiated at 10 Gy and 20 Gy resulted in a significant fall of the synthetic index, but the values did not fall to, nor below the normal level. On the other hand, DEN treated group administered with liposomal AK-2123 and irradiated at 10 Gy and 20 Gy showed relatively lower synthetic indices in a dose dependent manner.

Table 8 DNA synthesis measurement by BrdU labeling assay.

Groups	n	$\bar{x} \pm$ SD	*P*
Normal	10	0.0714 ± 0.013	—
DEN-treated	10	0.5087 ± 0.062	< 0.0001
DEN+Free AK-2123	6	0.5077 ± 0.063	N.S.
DEN+Liposomal AK-2123	6	0.4687 ± 0.060	N.S.
DEN+10 Gy.	6	0.0363 ± 0.005	< 0.0001
DEN+20 Gy.	6	0.0203 ± 0.004	< 0.0001
DEN+Free AK-2123+10 Gy.	10	0.1522 ± 0.020	< 0.0001
DEN+Liposomal AK-2123+10 Gy.	10	0.0886 ± 0.011	< 0.0001
DEN+Free AK-2123+20 Gy.	10	0.1496 ± 0.014	< 0.0001
DEN+Liposomal AK-2123+20 Gy.	10	0.0773 ± 0.011	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

3.4.5 Effects Of Radiation And AK-2123 On RNA Synthesis

Results obtained from RNA synthesis study are shown in Figs. 17 & 18 and the numerical values are shown in Table 9.

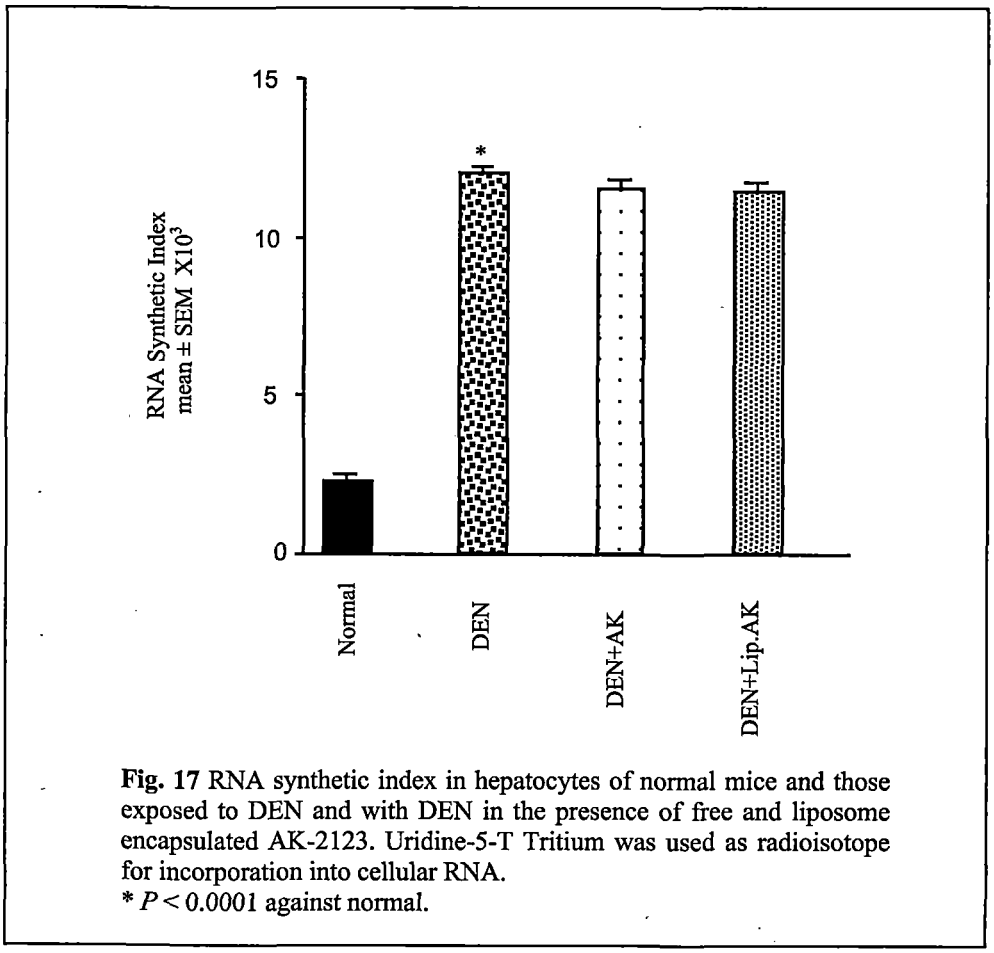


Fig. 17 RNA synthetic index in hepatocytes of normal mice and those exposed to DEN and with DEN in the presence of free and liposome encapsulated AK-2123. Uridine-5-T Tritium was used as radioisotope for incorporation into cellular RNA.
* $P < 0.0001$ against normal.

Table 9 RNA synthesis measurement by [^{3}H]-U incorporation assay.

Groups	n	$\bar{x} \pm$ SD	*P*
Normal	12	2372.525 ± 401	—
DEN-treated	12	12032.93 ± 577	< 0.0001
DEN+Free AK-2123	6	11572.58 ± 637	N.S.
DEN+Liposomal AK-2123	6	11447.35 ± 711	N.S.
DEN+10 Gy.	6	1111.800 ± 63	< 0.0001
DEN+20 Gy.	6	801.1111 ± 110	< 0.0001
DEN+Free AK-2123+10 Gy.	10	6541.010 ± 383	< 0.0001
DEN+Liposomal AK-2123+10 Gy.	10	4236.000 ± 284	< 0.0001
DEN+Free AK-2123+20 Gy.	10	5377.450 ± 207	< 0.0001
DEN+Liposomal AK-2123+20 Gy.	10	3293.166 ± 387	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

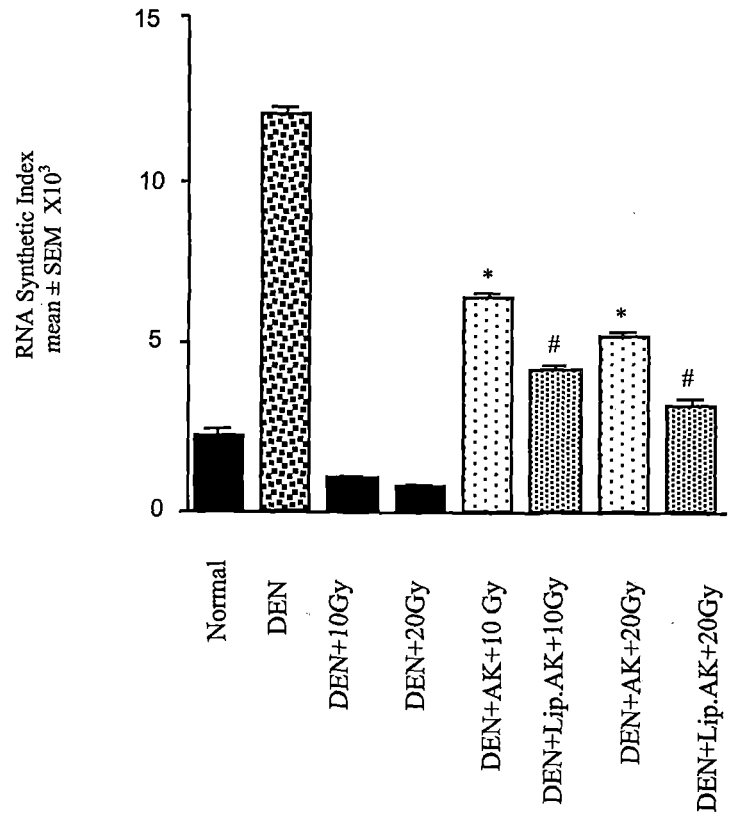


Fig. 18 RNA synthetic index in hepatocytes of normal mice and those exposed to DEN followed by irradiation (10 Gy & 20 Gy) in the presence of free and liposome encapsulated AK-2123. Uridine-5-T Tritium was used as radioisotope for incorporation into cellular RNA.
* $P < 0.0001$ against DEN.
# $P < 0.0001$ against DEN & DEN + AK-2123 + radiation.

Fig. 17 shows a distinct elevation of the RNA synthetic index following DEN treatment

registering an increase of 5.07 folds. There was no significant change in the index following administration of either free AK-2123 or liposomal AK-2123 alone. But as obvious from Fig.18, the index went down significantly, going down the normal level, upon exposure to radiation alone at 10 Gy and 20 Gy. The DEN treated group exposed to free AK-2123 and liposomal AK-2123 and irradiated at 10 Gy and 20 Gy showed a significant decreased in the index values, with the latter showing a better response than the former. The numerical values are shown in Table 9.

3.4.6 Effects Of Free And Liposomal AK-2123 On WBC

Fig. 19 shows the total WBC counts in normal and DEN treated mice in presence of free and liposome encapsulated AK-2123. The total WBC count was approximately two times higher in the DEN treated group than that of the normal group (Table 10). Administration of free AK-2123 to the normal and DEN treated groups of mice resulted in a significant decrease in the total WBC counts in both the cases. Administration of liposomal AK-2123 also resulted in a decrease in the total WBC counts, but the effect was not as significant as in the case of free AK-2123 (Fig. 19, Table 10).

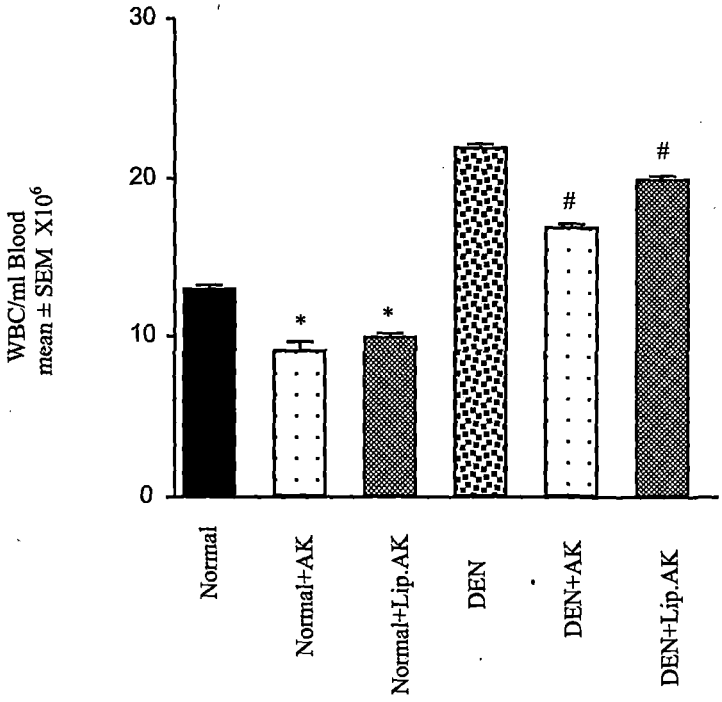


Fig. 19 Total WBC counts in the blood of normal mice and those treated with DEN alone, and with DEN in the presence of free and liposome encapsulated AK-2123.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against DEN.

Table 10 Total WBC counts (number of cells/ml blood).

Groups	n	$\bar{x}$	SD	*P*
Normal	6	13.058×10^6	0.45961	—
DEN-treated	6	22.057×10^6	0.45961	< 0.0001
Normal+Free AK-2123	6	9.3520×10^6	1.13137	< 0.0001
Normal+Liposomal AK-2123	6	10.082×10^6	0.45962	< 0.0001
DEN+Free AK-2123	6	16.919×10^6	0.60104	< 0.0001
DEN+Liposomal AK-2123	6	19.996×10^6	0.42426	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

3.4.7 Effects Of Free And Liposomal AK-2123 On RBC

In the DEN treated group the total RBC counts was approximately two times higher as compared to the normal mice (Fig. 20, Table 11). Following administration of free

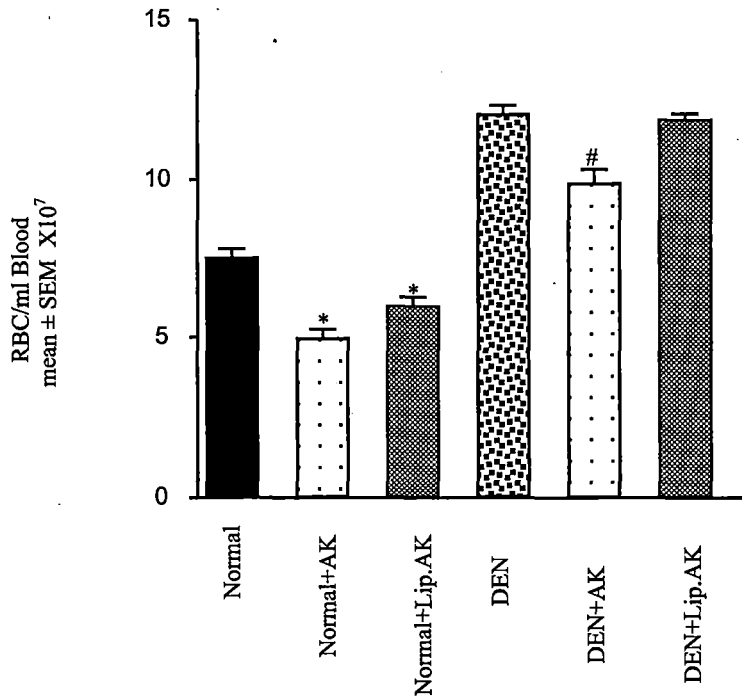


Fig. 20 Total RBC counts in the blood of normal mice and those treated with DEN alone, and with DEN in the presence of free and liposome encapsulated AK-2123.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against DEN.

and liposomal AK-2123 in the normal group there was a significant fall in the total RBC counts. The effect was less pronounced in case of liposome encapsulated AK-2123. In the case of DEN treated groups of animals, free AK-2123 administration also showed a significant fall in the total RBC counts, but it was not as significant as compared to the DEN treated group of mice alone (Fig. 20; Table 11).

Table 11 Total RBC counts (number of cells/ml blood).

Groups	n	$\bar{x}$	SD	*P*
Normal	6	7.540×10^7	0.23094	—
DEN-treated	6	12.14×10^7	0.22805	< 0.0001
Normal+Free AK-2123	6	5.040×10^7	0.23094	< 0.0001
Normal+Liposomal AK-2123	6	6.039×10^7	0.22805	< 0.0001
DEN+Free AK-2123	6	9.945×10^7	0.37527	< 0.0001
DEN+Liposomal AK-2123	6	11.89×10^7	0.22805	N.S.

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

3.4.8 Effects Of Free And Liposomal AK-2123 On Haemoglobin

Haemoglobin level in DEN treated group of mice was elevated by 1.103 folds as compared to that of the normal group of mice (Fig. 21, Table 12). When free AK-2123 was administered to normal group of mice, a distinct decline in the haemoglobin level was observed; while that, administered with liposomal AK-2123 showed no significant difference in the haemoglobin level as compared to the level of the normal groups. However, DEN treated group of mice upon free AK-2123 and liposomal AK-2123 administration, showed significant fall in the haemoglobin levels in both the cases. The effect was less pronounced in case of liposomal AK-2123 (Fig. 21, Table 12).

Table 12 Haemoglobin content in blood (mg/ml).

Groups	n	$\bar{x} \pm$ SD	*P*
Normal	6	107.685 ± 3.530	—
DEN-treated	6	118.796 ± 4.243	< 0.0001
Normal+Free AK-2123	6	96.2024 ± 1.202	< 0.0001
Normal+Liposomal AK-2123	6	107.026 ± 3.432	N.S.
DEN+Free AK-2123	6	82.4424 ± 1.640	< 0.0001
DEN+Liposomal AK-2123	6	94.1413 ± 2.128	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

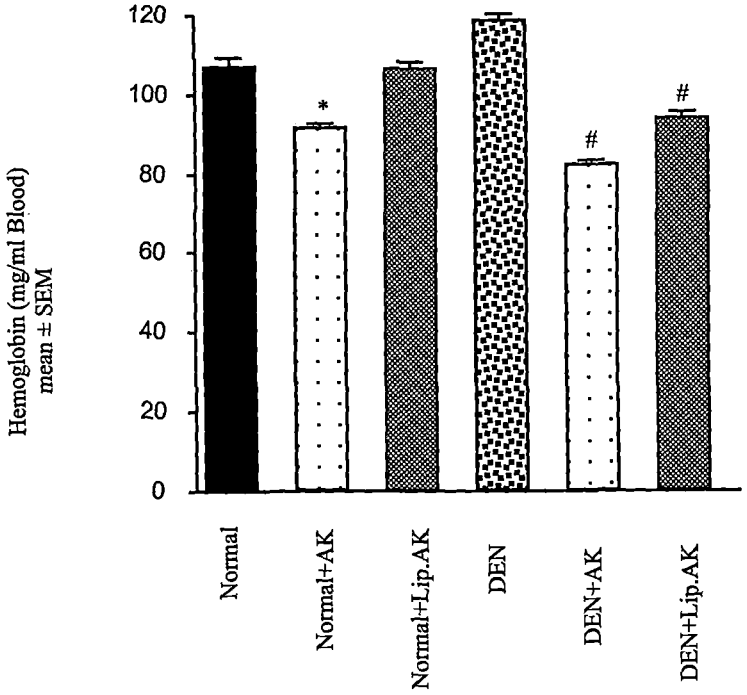


Fig. 21 Level of blood haemoglobin in normal mice and those treated with DEN alone, and with DEN in the presence of free and liposome encapsulated AK-2123.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against DEN.

3.4.9 Effects Of Free And Liposomal AK-2123 On Total Blood Protein

The total protein content was estimated by the method of Bradford[26]. The groups of mice that have undergone complete DEN treatment exhibited higher blood protein content than their normal counter parts (see Table 13, Fig. 22).

Table 13 Total protein content in whole blood (mg/ml).

Groups	n	$\bar{x} \pm SD$	*P*
Normal	6	10.0006 ± 0.213	—
DEN-treated	6	11.5500 ± 0.165	< 0.0001
Normal+Free AK-2123	6	9.18705 ± 0.291	< 0.0001
Normal+Liposomal AK-2123	6	9.98833 ± 0.212	N.S.
DEN+Free AK-2123	6	10.4140 ± 0.213	< 0.0001
DEN+Liposomal AK-2123	6	10.9125 ± 0.220	< 0.0001

n = No. of Observations, $\bar{x}$ = Mean, SD = Standard Deviation, *P* = Level of Significance, N.S. = Not Significant.

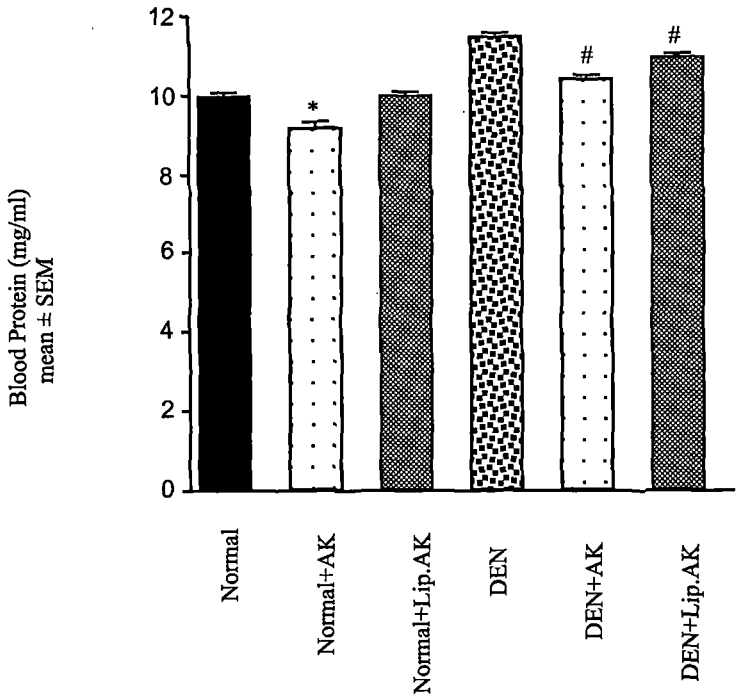


Fig. 22 Total protein content in the whole blood of normal mice and those treated with DEN alone, and with DEN in the presence of free and liposome encapsulated AK-2123.
* $P < 0.0001$ against normal.
# $P < 0.0001$ against DEN.

Free AK-2123 administration to normal mice showed a significant fall in the total protein; while upon liposomal AK-2123 administration, the protein content remained unchanged. However, administration of AK-2123 in it's free or liposome encapsulated forms to the DEN exposed mice resulted in decrease in total protein content that was statistically significant (Fig. 22, Table 13).

4 DISCUSSION

Chemically induced carcinogenesis process begins with "initiation" in which a chemical carcinogen irreversibly mutates and introduces some alterations in the segments of genomes. DEN, an established hepatocarcinogen, along with C_2H_5OH as promoter, were used to induce carcinogenesis in experimental mice. The induction protocol triggered the initiation process of carcinogenesis, which was achieved in a short course of time and was much pronounced in comparison to DEN alone. Reports available state that initiation of carcinogenesis is induced only when the carcinogens are coupled with a proliferative stimulus, especially in the liver[155]. Proliferative stimuli such as those exerted by partial hepatectomy promote the initiation process[153, 73]. It has also been reported that several carcinogens that normally do not induce liver cancer in adult animals become hepatocarcinogenic when coupled with liver cell-proliferative stimulus such as partial hepatectomy[36, 162].

In the present work, the hepatocytes were initiated by an intraperitoneal DEN dose of 150 mg/kg body weight and the animals were left to recover for two weeks. This dose causes centrilobular necrosis and, as one might expect, a regenerative hyperplasia extending almost over the whole two week period. It is assumed that this procedure generates numerous initiated hepatocytes, which can resist the cytostatic effects of subsequent administered carcinogens[93]. Cell proliferation has often been associated with the carcinogenic process in a variety of conditions. In liver, in particular, it appears that carcinogens induce initiation only when their administration is coupled with a proliferative stimulus[155]. Although the mechanism by which cell proliferation induces initiation is not clear, it's involvement in the occurrence of events such as fixation of a miscoding or a non-coding lesion, recombination, or hypomethylation in nucleotides of newly made DNA may explain it's role in this process[1, 154]. Cell proliferation is involved not only in the initiation step of chemical carcinogenesis but also in the promotion phase. Evidences suggest that proliferative stimuli such as those exerted by partial hepatectomy or necrogenic doses of CCl_4 promote the appearance of nodules and hepatocellular carcinomas following initiation with carcinogens[8, 9, 153, 73]. In fact, it has been reported by Columbano *et al.*[2], that irrespective of the type of initiation regimens used, preneoplastic hepatocytes were seen only in rats that received the carcinogen coupled with a cell-proliferative stimulus such as partial hepatectomy. In addition, in most of the experiments, carcinogen was given following partial hepatectomy, and often a non-necrogenic dose becomes necrogenic to liver when given to a partially hepatectomized rat[2]. Several procedures have been

described[137, 142], which selectively and rapidly stimulate the growth of the carcinogen-altered cells into foci islands of hepatocytes following initial treatment with a carcinogen, which resulted in an increased cancer incidence[118, 141]. Thus, initiated cells can be defined as those carcinogen-altered cells, which can be stimulated to grow into foci. These observations suggest that an exogenous cell proliferative stimulus is a critical factor in the initiation of liver carcinogenesis and that cell proliferation is an important step in the induction process[2].

In a hepatectomized condition, cell proliferation is triggered in order to compensate for the loss of the cells and within one week about 80% of the liver is restored. In the pre-hepatectomized initiated hepatocytes, this type of cell proliferation defined as compensatory regeneration is positively correlated with the initial phase of chemical carcinogenesis[2, 155]. It seems likely that initiating event involves some form of carcinogen-DNA interaction and subsequent damage. Neither chemical promotion nor surgical treatment alone is sufficient for cancer induction in the adult animal[169].

The activities of the marker enzymes and the synthetic indices of the nucleic acids underwent a marked elevation following DEN treatment (Figs. 2, 3). This indicates that the hepatocytes had undergone cellular transformation, and this is further substantiated by the histological studies (Fig. 4), which clearly showed the alteration of the hepatocytes in the DEN treated mice.

The GGT activity, which is otherwise negligible in adult mice, was significantly increased upon DEN administration. It is known that the reactivation of GGT is achieved only in cases of spontaneous or experimentally induced hepatocarcinogenesis. The alteration of GGT activity is dependent on the total dose of carcinogen administered. However, due to different methods the values are not always directly comparable. A 2 to 5-fold elevation can be achieved by a single dose of a potent carcinogen. After a chronic treatment and until the development of hepatocellular carcinomas (HCC), a 20 to 60-fold increase in liver GGT was reported which is comparable to the activity measured in fetal rat liver[27, 31]. In fact, it has been reported that GGT activity in liver that has undergone carcinogenic transformation can be greatly altered and elevated by as high as 40 to 100 folds than that of normal liver[113, 131]. The level of GGT, however seems to be independent of the malignancy of the tumour, as all transplantable hepatomas investigated showed a high

GGT activity, regardless of the degree of differentiation and the rate of proliferation[22]. As mentioned earlier that adult mice and rat hepatocytes have very negligible or, are almost totally devoid of GGT activity, the reappearance of the marked enzymatic activity in treated adult animals, indicate the reactivation of the enzyme on cancerization of hepatocytes, and this reappearance is often referred to as a 'carcino-embryonal' features[22]. This high GGT level is characteristic of carcinogenic transformation and not rapid proliferation of hepatocytes[33]. A strikingly high and rapid elevation of liver and serum GGT has also been reported to occur after treatment with a hepatocarcinogen[22]. Many authors have advanced the hypothesis that GGT is a general feature of all induced rat liver tumours, and that all chemical hepatocarcinogens activate GGT in rat liver[44].

Alteration in plasma membrane characteristics is a known feature of cellular transformation. AChE was chosen as the second marker enzyme because it is a membrane-bound enzyme and thus, any cellular transformation brought upon under the influence of the administered DEN could therefore, be monitored by assaying AChE activity. As expected, there was an elevation of AChE activity in the supernatant fraction of liver following DEN administration (Fig. 2B). This elevation of the AChE activity indicates that DEN administration had brought about some changes in the membrane of the hepatocytes.

Clinically, cancer is confirmed based on biopsy and histological verification of the disease. Therefore, histological study was also taken up for monitoring the induction process. The microphotographs, which were shot at the same magnification (**M** X40), exhibited a vast difference in the morphology of the liver in normal and treated situations (Fig.4). The treated liver seemed to have undergone a lot of alterations, which resulted in the following characteristics: -

(a) There was a loss of regular arrangement of the DEN treated hepatocytes which were distributed in a somewhat disarrayed fashion, the architecture of the supporting matrix seemed coarser, disorganized and disoriented as compared to the smoother, more uniform supporting matrix and distinct and defined arrangement of the cells in the normal condition.

(b) The DEN treated hepatocytes underwent marked variations in their shapes and sizes. As is evident from the microphotographs, the cell sizes were much smaller with distorted and ill-defined cell shapes, compared to the normal hepatocytes which had a definite shape and their sizes were almost regular.

(c) There was an increase in cell number in the DEN treated case (Fig. 4).

(d) There was more intense staining observed in the nuclei of the treated hepatocytes. This may be due to the condensation of their genomic materials as compared to their normal counterparts (Fig. 4).
(e) A large number of the DEN treated hepatocytes were multinucleated.

These morphological changes in the liver cells are indications that the DEN treated mice were in a high and active state of division. This suggests a state of carcinogenesis in liver.

Since cancer is a state where the cells are no longer responsive to the normal growth controlling factors, so uncontrolled proliferation of cells occurs in such a condition. Cell proliferation has often been implicated in the carcinogenic process and in cell transformation induced by chemicals, radiation and viruses[36, 163]. Compelling evidence to support the role of cell proliferation in the carcinogenic process has come from the observation that several carcinogens that normally do not induce liver cancer in adult animals become hepatocarcinogenic when given in a single dose when coupled with liver cell-proliferative stimulus such as partial hepatectomy[36, 162]. In order to keep up with this uncontrolled cell division, the nucleic acids also have to step up their rates of syntheses and therefore, accordingly result in an increase in their synthetic indices. This explains the increase in the synthetic indices of DNA, assayed by BrdU labeling method and that of the RNA assayed by [^{3}H]- U incorporation method. The DNA synthetic index was enhanced by 7.12 folds as compared to that of the normal (Fig. 3); while the synthetic index of RNA was elevated by 5.07 folds (Fig. 3). It has been demonstrated that carcinogens, either by themselves or after being activated, interact with DNA[86, 43, 139]. Evidence was also obtained to indicate that it is the DNA replication during cell proliferation, which is important for the induction of the initiated hepatocytes. In addition, recently, it has been shown that liver DNA with carcinogen-induced lesions replicates *in vivo* [11, 35, 172] and that such newly made DNA synthesized on a carcinogen-damaged template is stable[172]. Although the mechanism by which cell proliferation exerts it's unique effect in the initiation phase of carcinogenesis is not clearly understood, replication of DNA with carcinogen-induced lesions prior to repair offers an attractive mechanism by which carcinogen-induced critical damage may be appropriately transcribed and fixed into the newly made DNA and thus result in an initiated cell[2].

Furthermore, the SDS-PAGE profile of the perchloric acid liver extract showed that there was a significant over expression and under expression of several surface membrane glycoproteins (Fig. 5). This indicates that there was a differential expression of the liver surface membrane glycoproteins in DEN treated mice. The perchloric acid extraction method specifically extracts membrane glycoproteins and this observation indicates that DEN inflicted major alterations in the cell membrane glycoproteins as is evident from the varying intensity of the protein bands in the SDS-PAGE gel (Fig. 5). These changes could be involved in causing distortions and alterations of the cell membranes following DEN treatment (Fig. 4).

In DEN treated mice, the elevation of the marker enzyme activities, the changes in the morphology of liver cells and the differential expressions of surface membrane glycoproteins of the hepatocytes are direct evidences of cellular transformation. Since during this transformation RNA synthetic index was significantly enhanced, it may be suggested that DEN caused alterations in gene expression.

After having established cellular transformation, regression studies were carried out by administration of AK-2123, a radiomodifier, both in it's free and liposome encapsulated forms, combined with whole body γ-irradiation. The parameters monitored were marker enzyme activities, nucleic acid synthetic indices in liver tissue, and in blood, the RBC and WBC counts, haemoglobin content, and the total blood protein. Before performing the regression studies, the % entrapment efficiency of the liposome for AK-2123 was determined.

For the determination of % entrapment efficiency of liposomes, LASER Raman spectroscopy was chosen over other spectroscopic techniques since the UV absorption spectrum of AK-2123 gives a very broad band with no specific absorption maximum. IR spectroscopy was ruled out since AK-2123 is insoluble in organic solvents. In contrast, the presence of a free nitro group (- NO_2) in AK-2123 is ideally suited for the use of LASER Raman spectroscopy since - NO_2 is known to show a characteristic Raman peak at 1313 cm^{-1}.

The results showed that different concentrations of AK-2123 had characteristic peak for $-NO_2$ at 1313 cm^{-1} and the peak areas increased proportionately with the increase in the concentration of AK-2123. As is evident from Fig. 7, AK-2123 at a concentration of 2.5 mg/ml showed the smallest peak area; while at 20 mg/ml it showed maximum peak area. Peak 'U' is that of the unknown AK-2123 sample. The peak area of 'U' when interpolated in the standard plot and calculated, gave a percent entrapment efficiency of 40.6 %, which is reasonably high. Therefore, as expected from the fact that $-NO_2$ has a characteristic Raman Peak at 1313 cm^{-1}, AK-2123 also gave a peak at this particular wave-number by virtue of the presence of a free $-NO_2$ in it's chemical composition and thus, enabled the determination of the % entrapment efficiency of the prepared liposomes which otherwise was not possible to study by other spectroscopic techniques.

An animal, either normal or treated, upon exposure to whole body γ-irradiation experiences a loss in the number of viable cells. It has been observed that the greater the radiation dose to which the animal is exposed, the greater is the reduction in the cell number. This suggests that damage to both healthy as well as transformed cells are caused by radiation in a dose dependent manner. This property of radiation has been a serious deterrent in the successful application of radiation in cancer treatment. However, radiomodifiers can influence the effect of radiation by either enhancing the sensitivity of the transformed cells to, or shielding the normal cells from the effects of radiation. Administration of AK-2123, a radiomodifier, produces such effects. The assay of % cell viability at different doses of radiation showed that AK-2123 shielded the cells from the harmful effects of radiation from 8 Gy onwards and that, this protective effect was relatively more pronounced in case of AK-2123 encapsulated in liposomes at the radiation doses of 10 Gy and 20 Gy (Fig. 9). As seen from the calculated VMF of the hepatocytes following whole body γ–irradiation, free AK-2123 exhibited a VMF of 1.37 at 10 Gy and 1.54 at 20 Gy; while liposomal AK-2123 showed a VMF of 1.53 and 1.74 at 10 and 20 Gy respectively (Fig. 10). Presence of free AK-2123 improved the chance of survival of the cells at 10 and 20 Gy, and AK-2123 when encapsulated in liposomes offered better protection in relation to free AK-2123 at the same radiation doses as is obvious from their VMF at different radiation doses (Fig. 10).

The regression studies by assays of marker enzymes and cell proliferation upon exposure to radiation, administration of free AK-2123 and liposomal AK-2123 alone, and coupled with radiation exposure exhibited a common trend in all the cases. Therefore, a general common explanation can be sought for all these cases.

The results in all the cases indicate that radiation alone inflicted normal and transformed cell destructions which is signified by the very low levels of marker enzyme activities (Figs. 12, 14) and nucleic acids synthetic indices (Figs. 16, 18). The presence of AK-2123 either in it's free or liposome encapsulated form, along with radiation, inhibited this effect of radiation on the cells. Both forms of AK-2123 when present alone exhibited no statistically significant effects (Figs. 11, 13, 15 & 17). This observation indicates that AK-2123 alone has no toxic effect on the liver as well as on the nucleic acids. However, in presence of radiation, AK-2123 sensitized the transformed cells to radiation and hence resulted in the fall of enzyme activity levels and the rates of nucleic acids syntheses. Furthermore, animals treated with liposome encapsulated AK-2123 were seen to be better responding to radiation indicating that AK-2123 when encapsulated in liposomes exhibited better radiosensitizing effect than free AK-2123. This observation can be explained from the fact that liposomes have an inherent preferential migration towards tissues rich in reticuloendothelial cells like the liver[46]. Therefore, a larger proportion of AK-2123 reaches the target tissue, the liver in this study, and thus, liposome encapsulated AK-2123 exhibited higher radiosensitizing property than it's free counterpart. Of all the AK-2123 administered groups combined with radiation exposure, the groups administered with liposome encapsulated AK-2123 and exposed to 20 Gy radiation seems to respond much better than the rest of the groups and this is evident from it's close proximity to the normal levels in the marker enzymes assays and in the rates of the nucleic acids syntheses (Figs. 12, 14, 16, & 18).

Since the haemopoietic system is also a predominant target organ of chemical carcinogens, therefore, the effects of free and liposome encapsulated AK-2123 on certain blood parameters, namely, the haemoglobin content, number of blood cells and the protein content in whole blood were studied. The observations derived from these studies (Figs. 19-22) showed that DEN treatment caused an increase in the parameters studied, indicating that transformation had occurred upon DEN treatment. The result obtained from the study of free AK-2123 administration suggests that AK-2123 might have some level of toxicity,

which might be responsible for bringing down the values of the aforementioned parameters. In addition to this, the action of AK-2123 on the vascular system, as already mentioned in the introduction section, might also contribute to this suppressive effect. Liposome encapsulated AK-2123 showed no such drastic suppressive effects in all the cases. It may be due to it's inherent property of preferential migration towards the reticuloendothelial cells rich tissues which limits it's free distribution elsewhere. Consequently, it's free contact with blood is restricted. This may prevent the encapsulated AK-2123 from imparting it's toxic and suppressive effects in the blood unlike in the case of free AK-2123. Hence, encapsulated AK-2123 exhibited lesser toxic and suppressive effects on the parameters under study.

Throughout the regression studies, it is interesting to note that the level of the marker enzymes and the rates of nucleic acids synthesis did not fall to the normal levels or below, in spite of the presence of the radiosensitizer, AK-2123, which was supposed to sensitize the transformed cells and get them killed by radiation. When AK-2123 was first introduced in 1985 by Kyoto University group, it was proposed to be a hypoxic cell radiosensitizer. Several reports available on this compound are based on *in vitro* studies. However, recent studies suggest that AK-2123 may not only be a purely hypoxic cell radiosensitizer, and it has been shown that this compound exhibited distinct variations in it's radiosensitivity when present in oxic and hypoxic conditions of irradiation[157, 70]. Kagiya[157] and Imamura *et al.*[70], independently reported that the radiosensitivity enhancement ratios of AK-2123 for V79-379A cells under oxic and hypoxic conditions were different. These results show that AK-2123 has lower radiosensitivity in oxic conditions of irradiation. Balmukhanov *et al.*[130], reported that oral administration of AK-2123 did not show any sensitizing effect on radiation therapy of cervix and lung cancers. He also reported that AK-2123 was not effective in the radiation therapy of patients with larynx and tongue cancers as well. The resorption was observed in only 1 out of 9 patients taken in the trial[130]. It may be noted that these mentioned organs especially the lungs, are relatively oxygen rich. This may explain for the not so successful role of AK-2123 in such organs/cancers in oxic conditions.

Hebbar and George[132] assessed the radiation induced lipid peroxidation in erythrocyte ghost membrane in the absence or presence of AK-2123. They found that AK-2123 alone did not exert any significant effect, but it markedly reduced the radiation-induced lipid peroxidation when present at all concentrations in oxygenated conditions of

irradiation. Radiation induced normal tissue damage namely, muscle lipid peroxidation, was also diminished following administration of the drug in *vivo* (Swiss mice). These observations suggested that under aerobic conditions, AK-2123 protects against radiation-induced membrane oxidative damage. The report shows that AK-2123 affords remarkable radioprotection against γ –ray induced membrane peroxidative damage both *in vitro* and *in vivo* in oxic conditions of irradiation. The results suggest that AK-2123 has a dual function-as a hypoxic cell radiosensitizer and also as a radioprotector against radiation-induced membrane peroxidative damage in well-oxygenated systems. It has been reported that this finding is invaluable in the radiotherapy of cancer[132]. This finding agrees with the report of Mitsuhashi *et al.*[112], who assessed the sensitizing effects of AK-2123 on the interaction of radiation, cisplatin and hyperthermia by clonogenic assay in the rat yolk sac cell line NMT-IR *in vitro* under aerobic conditions. They found that although a statistically significant increase in the G1 cell fraction was observed after AK-2123 treatment, no enhancing effect of AK-2123 on radiation, cisplatin and heat response curves was detected under these conditions *in vitro*[112].

It is known that ionizing radiation promotes the membrane depolarization and Ca^{2+} uptake in cells of different lines[102, 136] and it has been reported recently that the effects of radiation on phagocyte cells can be connected with the change of the membrane processes (ion permeability, viscosity etc) which influence the NADPH-oxidase activity, superoxide anion secretion and degranulation processes[69]. Studies of the influence of γ-irradiation and radiosensitizer AK-2123 on secretion of reactive oxygen species of neutrophils of gastric cancer patients showed that the addition of AK-2123 depressed the early Phorbol Myristate.Acetate (PMA) stimulated chemiluminescence response to γ-irradiation. The results obtained suggest that this effect is connected with the influence of AK-2123 on ion canals of neutrophils and may prevent the radiation-induced damage of blood cells. This observation led Schepetkin[69] to conclude that AK-2123 inhibits the priming effect of ionizing radiation on neutrophils, likely due to the ion transport inhibition. Therefore, it appears that polarization/depolarization of the cell membranes may play a role in depressing the radiosensitization effect of AK-2123. The inhibitory effect of AK-2123 on the ion transport system is further supported by the report of Konovalova *et al.*[81], which shows that AK-2123 has antimetastatic effect and this effect is determined by at least partial inhibition of active transport of calcium ions across sarcoplasmic reticular cells affected by Ca^{2+}

dependent Mg^{2+}activated ATPase. Thus, it is obvious that the effect of AK-2123 on the membrane transport system also plays a decisive role on the properties[81] exhibited by AK-2123.

In the present study, the inability of GGT and AChE enzymes or the rate of nucleic acids syntheses, to regain the normal levels after the AK-2123 combined with radiation treatment, may be because the mode of irradiation carried out for the entire study was in aerobic conditions. It must be noted that no attempt was made to achieve a hypoxic condition in the tissue at any point of the entire course of study. Moreover, it must also be noted that whatever alterations that had taken place by the induction protocol, were only cellular transformations, as is evident from Fig. 4, without affecting the oxygen supply and circulation. In other words, the DEN treated liver and the hepatocytes were in conditions close to normal aerobic conditions. The induction process did not reach a stage of large lumpy tumor masses, within which there is severe disruption of oxygen supply and where the microenvironment is intrinsically depleted of oxygen and nutrients. The effect of AK-2123 on the membrane ion transport system of the transformed cells may also contribute to the observations obtained in this study. Therefore, in oxic conditions of irradiation, AK-2123 shows a relatively lower and weaker radiosensitizing effects.

The initial laboratory investigations on radiomodifying properties of AK-2123 were conducted *in vitro* systems. Based on these studies, AK-2123 was categorized as a hypoxic cell radiosensitizer. This encouraged putting AK-2123 in limited clinical trials. The results of these *in vivo* studies were not in line with those of *in vitro* studies. A notable difference in the two conditions (*in vitro & in vivo*) is the level of oxygenation. Investigation that followed show that AK-2123 behaved differently under oxic and anoxic conditions. The present *in vivo* study also finds that AK-2123 showed rather weak radiosensitizing effects coupled with radioprotective effects on certain parameters. Since the mouse *in vivo* system is inherently oxic, but has different levels of oxygenation in different organ systems, the results are not unexpected. One clear conclusion to be drawn from this investigation is that AK-2123 may have differential radiomodifying effects in different tissues *in vivo*, and in different conditions of irradiation. Therefore, AK-2123 may be a suitable drug for chemo-radiotherapy of only certain types of cancer. Since solid tumors in late stage develop anoxic core, AK-2123 could be a potentially useful drug for their chemo-radiotherapy.

5 CONCLUSION

Thus, based on the results obtained from experiments conducted, it may be concluded that,

- Chronic exposure to NDEA combined with hepatectomy induced cellular transformations in the liver of Swiss albino mice, as substantiated by the pronounced alterations in the activities of the marker enzymes and the synthetic indices of the nucleic acids.
- NDEA treatment resulted in a distinct change in the nature of the hepatocytes such as the variations in the cell shape and size, appearance of more densely stained nuclei and multinucleated cells as elucidated in the histological studies.
- Upon NDEA exposure, the liver glycoproteins exhibited differential expressions. Some proteins were over expressed while others were under expressed as compared to their normal counterparts as revealed by the electrophoretic study.
- Radiation alone inflicted damage to both the transformed and normal cells, as reflected by the marked decline in the activities of the marker enzymes and synthetic indices of the nucleic acids.
- Liposomes obtained by the reverse phase evaporation method were relatively large in size and most of them were unilamellar.
- About 40% entrapment efficiency of AK-2123 into liposomes was achieved as determined by LASER Raman spectroscopy.
- AK-2123 alone, whether present in free or liposome encapsulated forms, had no toxic effect on the liver at a concentration of 200 mg/kg body weight.
- Liposomes retained the properties of the entrapped AK-2123. It's selective migration to the liver ensured that a major proportion of the drug reached the liver intact, as is evident from the levels of the activities of the marker enzymes and nucleic acid synthetic indices, which were in the proximity of the normal levels. This is further supported by the observation that AK-2123 when encapsulated in liposomes showed lesser suppressive effects on the haemopoietic parameters studied, as compared to it's free form.
- AK-2123 in the oxic conditions of irradiation exhibited lesser radiosensitizing effects as compared to the hypoxic mode of irradiation thereby, affording radioprotective effects.

It is seen from the fact that none of the transformed groups of animals administered with either free or liposome encapsulated AK-2123 and followed by exposure to radiation in oxygenated conditions, regained their actual normal levels of enzyme activities and nucleic acids' synthetic indices, nor fell below the normal levels.

- Overall, AK-2123 encapsulated in liposomes afforded better radiomodulation than free AK-2123.

Therefore, there is scope for improving cancer therapy. One of the possibilities may be the use of radiation coupled with radiomodulatory drugs in combination with a drug delivery system. This combination offers ample scope for research and investigations and hence, the prospects of improving existing cancer therapies.

APPENDIX Abbreviations

[^{3}H]-U	Uridine-5-T Tritium
ABTS	2,2'-azino-di-[3-ethylbenzthiazolin-sulfonat]
AChE	Acetylcholine esterase
AK-2123	N- (2'-methoxyethyl)-2-(3"-Nitro-1"-triazolyl) acetamide
BrdU	5-Bromo-2'-deoxy-Uridine
BSA	Bovine serum albumin
CCl_4	Carbontetrachloride
$CHCl_3$	Chloroform
CH_3OH	Methanol
Chol.	Cholesterol
D.P.X.	Diesterene Plasticizer Xylene
DCP	Dicetyl phosphate
DMEM	Dulbecco's Modified Eagle Medium
DMPC	Dimyristoyl phosphatidyl choline
DPPC	Dipalmitoyl phosphatidyl choline
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
ELISA	Enzyme-linked immunoadsorbent assay
FCS	Fetal calf serum
g	Gravitational force
GGT	Gamma glutamyl transpeptidase
Gy	Gray
h	Hour
Hb	Haemoglobin
HMW	High molecular weight
kDa	Kilo Dalton
Lip.AK-2123	Liposome-encapsulated AK-2123
LMW	Low molecular weight
mA	Milliampere
MTP	Microtitre plate
DEN	N-Nitrosodiethylamine
NDMA	N-Nitrosodimethylamine
$(NH_4)_2SO_4$	Ammonium sulphate
NMU	N-methyl-N-urea
PAGE	Polyacrylamide gel electrophoresis
Egg PC	Egg Phosphatidyl choline
POD	Peroxidase
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl)-aminomethane
Triton X-100	(t-Octylphenoxypolyethoxyethanol)
U	Enzyme specific activity
V	Volt
Vh	Volt hour
VMF	Viability modification factor

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B.Sc. Chemistry in 1st Division	1991	North-Eastern Hill University
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Research Publications

1. Chakraborty, S., Rapthap, C., Alam, A., Srivastava, P.N. and Sharan, R. N., Liposome as a carrier for delivery for radiomodulatory drugs and it's advantages in chemo-radiotherapy. In Radiation, Radiomodifiers & Human Health. Ed. P. Uma Devi, K.S.Bisht & B.S.S. Rao. 1995, pp, 111-123. NISCOM, New Delhi, India.
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3. Anis Alam, Longkumer Imliwati, Chowphi Rapthap and Vinod Singh., Liposome Encapsulated Tumor-Associated Antigens Elicited Humoral And Cellular Immune Responses In Mice Bearing Tumor. Ind. J. Expt. Biol., 2000 (in Press).

Symposia & Workshops Participated

1. 17th Annual Convention Of Indian Association For Cancer Research, Chittaranjan National Cancer Institute, Calcutta,, January 21-24, 1998.
2. International Conference on Radiation Biology: DNA Damage, Repair and Carcinogenesis, North-Eastern Hill University, Shillong, April, 1998.
3. 19th Annual Convention Of Indian Association For Cancer Research, Amala Cancer Research Institute, Thrissur, January 21-23 2000.
4. Regional workshop on Trace Element Analysis FT-NMR Spectrometry, November 19-21, 1998. Regional Sophisticated Instrumentation Centre, NEHU, Shillong.
5. Regional workshop on FT-NMR Spectrometry, November 23-25, 1998. Regional Sophisticated Instrumentation Centre, NEHU, Shillong.
6. Regional workshop on Electron Microscopy (Entomology), November 27-28 1998. Regional Sophisticated Instrumentation Centre, NEHU, Shillong.