

CARCINOGENESIS RESPONSE MODULATION BY GELONIN ENCAPSULATED IN LIPOSOME

By



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Dedicated

To

My Beloved Mom, Dad & Teachers

THE NORTH-EASTERN HILL UNIVERSITY

Shillong, India

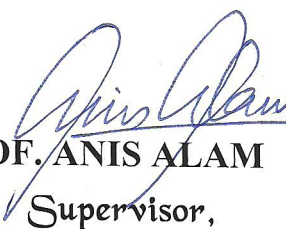
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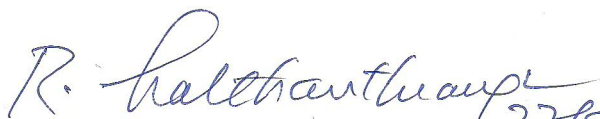
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ABBREVIATIONS

RIPs	Ribosome inactivating proteins
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
BMV	Brome mosaic virus
GGT	γ -Glutamyl transpeptidase
AChE	Acetylcholine esterase
GST	Glutathione-S-transferase
GSH	Glutathione (reduced)
BrdU	5-Bromo-2'-deoxyuridine
PBS	Phosphate Buffered Saline
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
CDNB	1-chloro-2, 4-dinitrobenzene
EDTA	Ethylene diamine tetra-acetic acid
TCA	Trichloroacetic acid
SDS	Sodium Dodecyl Sulfate
DMEM	Dulbecco's Modified Eagle Medium
FCS	Fetal Calf Serum
POD	Peroxidase
DPX	Diesterene Plasticizer Xylene
DBN	N-dibutyl nitrosoamine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
APS	Ammonium Persulfate
ELISA	Enzyme Linked Immunosorbent Assay
DTT	Dithiothretol
PE	Phosphatidyl ethanolamine
DPPC	Dipalmitoyl phosphatidylcholine
DCP	Dicetyl phosphate
PG	Phosphatidyl glycerol
Chol	Cholesterol

CHAPTER -1

INTRODUCTION

1.0.0 INTRODUCTION

Cancer is an ancient disease and has been known since human societies first learnt to record their activities. Although diseases of the heart and blood vessels are still the main cause of death in our ageing population, cancer is a major problem. Nearly everyone's life has been directly or indirectly affected by cancer. Therefore, its control or even better prevention by making use of improved systemic cancer therapies is urgently required.

Most scientists involved in cancer research believe that many cancer cases may be associated with the environment in which we live and work. In this context, the environment is anything that people interact with including lifestyle choices such as what we eat, drink, or smoke, natural and medical radiation, including exposure to sunlight, workplace exposures, drugs, socioeconomic factors that effect exposures and susceptibility and substances in the air, water and soil (Office of Technology and Assessment, 1981). Other factors that play a major role in cancer development are infectious diseases, aging and individual susceptibility such as genetic predisposition. We rarely know what environment factors and conditions are responsible for the onset and development, especially for cancers related to certain occupational exposures or the use of specific drugs.

Many experts firmly believe that much of the cancer associated with the environment may be avoided (Tomatis, L. *et al*, 1997). By changing our habits,

therefore, we should, in principle, be able to reduce drastically our chance of developing almost any given type of cancer. This is demonstrated most clearly by a comparison of cancer incidence in different countries; for almost every cancer that is common in a country, there is another country where the incidence is several times lower; and migrant population tend to take on the pattern of cancer incidence typical of the host country, implying that the differences are due to the environmental, not genetic factors. From such data it is estimated that 80-90% of cancer should be avoidable. Unfortunately, different cancers have different environmental risk factors, and a country that happens to escape on such danger is no more likely than other countries to escape the rest; thus the incidence of all cancers combined (among individuals of a given age) is similar from country to country. There are, however, some subgroups whose abstinent way of life does seem to reduce the total cancer death rate: the incidence of cancer among strict mormons in Utah, for example, is only about half that among Americans in general.

While such epidemiological observations indicate that cancer can be avoided, it remains difficult to identify the specific environmental risk factors to establish how they act. Some certainly operate as mutagenic tumour initiators, directly provoking genetic changes; others presumably serve as tumour promoters that help to enlarge the population of cells liable to progress, through further mutation, to full-blown cancer. The carcinogen in tobacco smoke, like the aflatoxin on tropical peanuts, probably belong mostly in the first category, while the reproductive hormones that circulates in a woman's body at different stages of her life may belong in the second category. The

importance of these hormones is indicated by the strictly correlations that exist between a woman's reproductive history and her risks of developing breast cancer; the hormones presumably affect cancer incidence through their influence on cell proliferation in the breast. It is possible that some factors act in still other ways, for example, by causing heritable epigenetic changes. Of course, it is not necessary to understand how cancer causing agents act in order to identify them and show how to avoid them. In this task cancer epidemiology has had some notable success and promises more to come. Simply by revealing the role of smoking, it has shown a way to reduce the total cancer death rate in North America and Europe by as much as 30%. The prevention of cancer is not only better than cure but seems also, given our present state of knowledge to be much more readily attainable.

Normal body cells grow, divided and die under control mechanisms. In early years of one's life, normal cells grow and divide more rapidly until an adult is attained and then body cells divide only to replace worn-out or dying cells and to repair the injuries. Cancerous cells result when a normal cell escaped normal growth-regulating mechanisms and continue to grow and divide thereby giving rise to clones of cells that can be expanded to a considerable size producing tumor.

A tumor, which does not invade the neighbouring tissue or spread to other part of the body, with very rare exceptions, are not life threatening, is benign. Malignant is one that started in one part of the body gets into blood stream or lymph vessels and spread and invades other parts of the body where they may replicate and result in the death of the patient. The term cancer refers to the full-blown malignant tumour.

Malignant tumors are broadly classified according to the embryonic origin of the tissue from which the tumour is derived. Malignant neoplasms arising from endodermal or ectodermal are called carcinomas, which constitute the major form. Malignant neoplasms that originate from connective tissue, muscle, cartilage, fats or bone are sarcomas. Leukemias and lymphomas are malignant tumours of hematopoietic cells of bone marrow.

After a quarter century of rapid advances, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function; both classes of cancer genes have been identified through their alteration in human and animal cancer cells and by their elicitation of cancer phenotypes in experimental models (Bishop, J. M. and Weinberg, R. A., 1996). The changes involved in the structure and function of cellular DNA by one of the following mechanisms: (a) direct interaction with DNA or RNA, which results in changes that are inheritable from cell line to cell line, (b) interference with enzymes that control DNA repair, replication, or transcription and, (c) interference with normal control mechanisms such as the histone that may contribute to DNA replication/synthesis.

Oncogenes; capable of causing cancer arise from the mutation of proto-oncogenes. 'Cancer genes' are component of the normal cellular genome whose activity is unleashed or augmented by carcinogens of various kinds and is then responsible for sustaining the undisciplined behaviour of cancer cells. Thus cancer genes are not alien

intruders but normal, indeed essential genes run amok; the damage done by a carcinogen turns friend to foe, perhaps by acting directly on cancer gene or perhaps by crippling a second gene that normally polices the activity of the cancer gene (Bishop, J. M. 1982).

1.2.0 CARCINOGENESIS

Carcinogenesis initiates and also can be induced experimentally by well characterized biological, chemical and physical agents (carcinogens). Some of the known carcinogenic agents are also natural causes of cancer in man and animals. Chemical carcinogenesis by tobacco smoke products is a major cause of common lung cancers. Physical carcinogenesis by ionizing radiations poses a potential world-wide threat in this nuclear age. Skin carcinogenesis by solar ultraviolet radiation is expected to increase even above its present high incidence as the ozone layer of the atmosphere undergoes depletion. Nevertheless, the specific causes of most common human cancers of breast, colon, rectum, lymph nodes, uterus, bladder, pancreas, bone marrow, stomach, and so on- remain unknown. Most types of cancer are difficult to eradicate and some, like hepatocellular carcinoma, are almost always fatal.

1.2.1 CHEMICAL CARCINOGENESIS

The discovery of chemical carcinogenesis was made by Sir Percival Pott (1713-1788), an English surgeon, who related the cause of scrotal skin cancer in a

number of his patients to a common history of occupational exposure to a large amount of coal soot as chimney sweeper when they were boys. Chemical carcinogens include a high diverse collection of chemicals substances both organic and inorganic chemicals, solid state materials, hormones, and immunosuppressants (Miller, E. C. *et al*, 1981). Chemical carcinogens of synthetic (man made) or natural origin are extremely diverse in structure without any common feature, and are classified into two categories: genotoxic and non-genotoxic.

Direct-acting or DNA-reactive, activation independent carcinogens that bind covalently to cellular genomic DNA is genotoxic and are mutagens. However, there are other carcinogens that required prior metabolism (activation dependent) to become carcinogenic (procarcinogens). The process whereby one or more enzyme-catalyzed reactions convert procarcinogens to active carcinogens is called metabolic activation. Any intermediate compounds formed are proximate carcinogens (there may be one or more), and the final compound that reacts with cellular components (e.g. DNA) is the ultimate carcinogen. A possible sequence can be displayed as follows:

Procarcinogens → Proximate carcinogens → Ultimate carcinogen

The procarcinogen itself is not a chemically reactive species, whereas the ultimate carcinogen is often highly reactive. An important generalization is that ultimate carcinogens are usually electrophiles (i.e. molecules deficient in electrons), which readily attack nucleophilic groups in DNA, RNA and proteins.

The metabolism of carcinogens and other xenobiotics involves monooxygenase and transferases. The enzymes responsible for metabolic activation of procarcinogens are principally species of cytochrome P450, located in the endoplasmic reticulum.

Carcinogens have been found to interact with the purine, pyrimidine, or phosphodiester groups of DNA. The most common site of attack is guanine, and the addition of various carcinogens to the N₂, N₃, N₇, O₆ and O₈ atoms of this base has been observed.

One of the characteristics of chemical or physical carcinogenesis is the usually extended period of time (latent period) between the contact with the carcinogens and the appearance of the tumour. Repeated exposures to the active agents are often, although not always, required which is another characteristic. The latency between exposure to a carcinogen and cancer formation is divisible into stages: initiation caused by agents that irreversibly and heritably alters the cell genome, promotion, the mechanism of which is not well understood, the latent period can be shortened and the tumour yield increased by treatment with certain 'promoting agents' which are not carcinogenic in themselves, or very weakly so. Progression, the third definable stage of neoplastic development, is separated from promotion in which high incidence of carcinomas can be produced by subsequent applications of a different initiating agent, suggesting a second event "second hit" in the induction of cancer. Thus, it appears those of the three stages of carcinogenesis- initiation, promotion and progression- initiation, most certainly, and progression most likely involve molecular genetic changes.

The actions previously described are those of agents, which react with cellular DNA and cause genomic alterations. As more and more chemicals are tested for carcinogenicity, a number are now being recognized as "non-genotoxic". The chemicals do not form stable covalent bonds with cellular DNA or other macromolecules. Possible mechanism for epigenetic effects include chronic tissue injury, hormonal imbalance, immunological effects, or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens (Weisburger, J. H., *et al*, 1984). This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants, and promoters. Examples of epigenetic carcinogens include nitrotriacetic acid, asbestos, phorbol esters, estradiol, azothioprine, etc.

1.2.2 NITROSOAMINE

N-Nitrosamines constitute one of the most interesting classes of chemical carcinogens. Early interest in the study of these compounds stemmed from findings that they present industrial occupational hazards. In recent years it has become increasingly evident, however, that N-Nitroso compounds are of great concern, not only to the industrial workers, but also to the population at large. With the recognition that N-Nitroso compounds can be readily formed from the precursors widely present in the environment, N-Nitroso compounds have emerged as one of the most important classes of the environment carcinogens. Experimentally, N-Nitroso compounds are among the most potent and versatile agents inducing tumors in every animal species tested; this renders these compounds highly useful tools for the study of chemical carcinogenesis.

They induced tumors in a wide variety of organs and tissues in a large number of animal species. The potency and targets specificities of an N-Nitroso compound depends not only on the structure of the chemical, but also on the dosage, treatment schedule, the route of administration, the animal species, and various other factors such as the age, sex and diet of the animals(Arcos, J. C., *et al* , 1982).

Nitrosoamines have been investigated for their carcinogenic and toxic properties since 1956 when the simplest alkylnitrosoamine, N-nitrosodimethylamine, at the time an important industrial intermediate, was shown to produce liver tumors in rodents. Since then more than 100 nitrosoamines have been shown to be carcinogens in the experimental animals, causing tumors mainly in the liver and also in a number of other tissues. The nitrosoamines require metabolic activation mediated by the cytochrome P 450 and flavin dependent oxidases. Such biotransformation yields electrophiles that readily alkylate nucleophilic sites in DNA (Arcos, J. C. *et al*, 1982).

1.2.3 MECHANISM OF ACTION

The main site of alkylation in nucleic acids is the N-7 position of guanine; N-1, N-3 and N-7 position of adenine, and N-3 position of cytosine, thymine and uracil have also been observed. It was hypothesized that O-6-alkylation of guanine leads to the inability of the guanine residue to undergo normal base pairing with cytosine and thus may lead to "transition" mispairing, resulting in mutation. The inability of the system to remove O-6-alkyl guanine from DNA, and therefore to reduce the level of this abnormal

purine in DNA before DNA replication, may be of critical importance for determining susceptibility to carcinogenesis by N-Nitroso compounds (Arcos, J. C. *et al*, 1982).

In addition to O-6-alkylation of guanine, the following reactions in nucleic acids have been suggested as playing an important role in carcinogenesis and mutagenesis by Nitroso compounds: (a) alkylation of phosphate moieties forming phosphodiester, which in DNA are chemically stable, and (b) alkylation at the N-3 position of guanine, the O - 4 position of thymine, the N-7 position of adenine, and the N-3 position of cytosine.

1.2.4 N-DIBUTYLNITROSOAMINE (DBN)

N-Dibutylnitrosoamine is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (International Agency for Research on Cancer: 1974, 1978, 1982, 1987). Interest in the study of the structure-activity relationships of DBN and related compounds arose mainly because of the somewhat unusual ability of DBN to induce bladder tumors. It was noted that dibutyl nitrosoamine induces tumors of the liver, esophagus, and bladder after oral administration. A shift of organ specificity was observed after sub cutaneous injections,

with the bladder as the main target organ. An extensive series of synthesis and carcinogenesis studies of various compounds related to DBN and its metabolites have revealed that they have markedly different carcinogenic effects. The metabolism of DBN is complex, because hydroxylation can take place at each of the four carbon atoms of the butyl chain.

N-Nitrosodibutylamine is pale yellow oil with a characteristic odour. It is soluble in water and miscible with hexane, dichloromethane, and many other organic solvents. It is sensitive to light, especially to ultraviolet light, and undergoes relatively rapid photolytic degradation. When heated to decomposition, it emits toxic fumes of nitrogen oxides (Hazardous Substances Data Bank, 2000; International Agency for Research on Cancer, 1978).

N-Nitrosodibutylamine has been detected in a variety of products as a result of the nitrosation of amines present in these products. It is present in soya bean oil at a concentration of 290 g / kg, and in smoke or cured meat at 0.2 to 3.9 g / kg (International Agency for Research on Cancer, 1978). It has also been detected in tobacco smoke at a concentration of 3 ng /cigarette. N-Nitrosodibutylamine may be formed from secondary or tertiary β -butylamines and quaternary ammonium salts by reaction with nitrosation agents, such as nitrite, in the stomach or during cooking processes. The computer estimated half-life of N-Nitrosodibutylamine in various phases is 2.8 days.

1.3.0 CHEMOTHERAPY OF CANCER

Nearly more than five decades, chemotherapy has been the main modality for systemic treatment of advanced or metastatic cancer (Chabner, B., 1990; Frei, E. III *et al*, 1997). Cancer chemotherapy is limited by intrinsic or acquired multi-drug resistance of tumor cells and toxicity to normal cells (Chabner, B., 1990; Frei, E. III *et al*, 1997). Although the effectiveness of chemotherapy can be increased by escalating the doses, the option for dose elevation is always restricted by toxicity of chemotherapeutic drugs.

The majority of clinically-used anticancer drugs are systemic anti-proliferative agents (cytotoxins) that preferentially kill dividing cells, primarily by attacking their DNA at some level such as synthesis, replication or processing. These cytotoxins have many advantages as anticancer drugs, especially the ability to kill large number of tumor cells. However, these drugs are not truly selective for cancer cells, and their therapeutic efficacy is limited by the damage they also cause to proliferating normal cells such as those in the bone marrow and gut epithelia. This is particularly true in the treatment of solid tumor, when the majority of the tumor cells themselves are not dividing rapidly. A more selective delivery of the cytotoxic agents to the primary tumors and their metastases would allow a dose escalation and reduce the peripheral toxicity (Magrath, T., 1994).

The concept of delivering cytotoxic drugs through a carrier such as tumor specific monoclonal antibodies, hormones, liposomes etc. to certain tumors has opened a new era in the development of potential therapeutic agents for cancer treatment. In the recent years, many ribosome-inactivating proteins (RIPs) from plants that catalytically



inactivate eukaryotic ribosome have been extensively used as therapeutic agents in cancer treatment (Chabner, B., 1990; Frei. E. III *et al*, 1997).

1.4.0 RIBOSOME-INACTIVATING PROTEINS (RIPs)

Ribosome-inactivating proteins (RIPs) are a group of naturally occurring plant proteins with a RNA-*N*-glycosidases activity which depurinate rRNA at a specific universally conserved position (i.e. cleavage of N-glycosidic bond of a specific adenine of 28S rRNA). The first two RIPs known were ricin and abrin. Dixon in 1887 (Dixon, T., 1887) was the first to suspect that the toxic principle of the castor beans was a protein. Shortly afterwards, Stillmark in Kobert's laboratory in Dorpat purified the protein, for which he proposed the name ricin, and attributed its toxicity to the property of agglutinating erythrocytes (Stillmark, H., 1888, 1889). Hellin also in Kobert's laboratory discovered the agglutinating properties of abrin (Hellin, H., 1891).

Interest in these toxins was revived as late as 1960, when Lin *et al.* reported that they were more toxic to tumor (Ehrlich ascites) than to normal cells (Lin, J., *et al*, 1970). These typical plant proteins receive a lot of attention in the biological and biochemical research because of their unique biological activities towards animal and human cells. In fact, for a long time the interest in RIPs focused on possible medical and therapeutical applications because several of these proteins were found to be more toxic to tumor cells than to normal cells, and hence offered a theoretical opportunity to develop antitumor drugs that selectively target tumor cells (Lin, J., *et al*, 1970). With the development of monoclonal antibodies as tools for identifying and targeting cell

surface markers, researchers gained the ability to couple antibodies to RIPs and thus deliver the toxic protein directly to specific cells. The potential for using RIPs as cell destructive agent in immunotoxins stimulated intense efforts to isolate and characterize such proteins from many different plant sources (Frankel, A. E, *et al*, 1996; Pastan, I., *et al*, 1991). Unfortunately, RIP-derived immunotoxins are not perfect clinical tools. For example, they are generally highly antigenic and promote immune responses in animals receiving prolonged treatment with the immunotoxins (Barbieri, L, *et al*, 1993). A second problem is with vascular leak syndrome, a deleterious side effect that limits clinical efficacy as a cancer therapy (Kreitman, R. J. 1999). Nevertheless refined approaches to inhibit toxicity are showing promises (Baluna, R. *et al*, 1999; Hirao, I., *et al*. 2000) and a number of clinical trials are ongoing (Frankel, A.E., *et al* 1996; Kreitman, R. J., 1999).

1.4.1 TYPES OF RIPs

RIPs are classified into three groups based on their physical properties (Mundy, J., *et al*, 1994). Type 1 RIPs, such as pokeweed antiviral protein (PAP), saporina from *Saporinia officialinis*) and barley (*Hordeum vulgare*) are monomeric enzymes, each with an approximately Mr of 30,000 (Asano, K., 1984; Barbieri, L., *et al*, 1993; Irvin, J. D., 1975). Type 1 RIPs were discovered in 1925 when Duggar and Armstrong (Duggar, B. M., *et al*, 1925) observed that the so-called *Phytolacca americana* antiviral protein (PAP) inhibits the transmission of tobacco mosaic virus (TMV) in plants. However, only in 1978 was PAP recognized as an inhibitor of protein synthesis (Dallal, J. A. *et al*,

1978). Many, but certainly not all, type 1 RIPs are antiviral proteins. Type 1 RIPs are not cytotoxic and do not behave as toxins because they are not able to cross the cell membrane on their own. Some specialized animal cells, however, can import type 1 RIPs by endocytosis and subsequently become sensitive to the RIP activity. To date, most RIPs that have been characterized fall into the type 1 class (Barbieri, L., *et al*, 1993).

Type 2 RIPs were discovered more than a century ago when Stillmark isolated the toxic principle from castor bean seeds. Ironically, the high toxicity of ricin was attributed to its agglutinating activity, which means that the carbohydrate binding activity of type 2 RIPs was recognized long before their enzymatic activities and their inhibitory activity on protein synthesis. Type 2 RIPs, like ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of approximate mw of 30,000 (Olsnes, S., *et al*, 1973; Olsnes, S., *et al*, 1982; Stripe, F., *et al*, 1978). One polypeptide with RIP activity (A-chain) is linked to a galactose binding lectin (B-chain) through a disulfide bond (Olsnes, S., *et al*, 1973; Olsnes, S., *et al*, 1982; Stripe, F., *et al*, 1978). The lectin chain can bind to galactosyl moieties of glycoproteins and /or glycolipids found on the surface of eukaryotic cells (Lehar, S. M., *et al*, 1994; Olsnes, S., *et al*, 1988; Sandvig, K., *et al*, 1976; Steeves, R. M., 1999; Swimmer, C., *et al*, 1992) and mediate retrograde transport of the A-chain to the cytosol (Beaumelle, B., *et al*, 1993; Olsnes, S., *et al*, 1982; Sandvig, K. *et al*, 1994; Van Deurs, B., *et al*, 1986). Once it reaches the cytosol, the RIP has access to the translational machinery and readily disrupts protein synthesis.

Type 2 RIPs owe their carbohydrate binding activity to the B-chain, which contains two or possibly three binding sites (Frankel, A. E., *et al*, 1996; Steeves, R. M., *et al*, 1999). Though the B-chain of different type 2 RIPs share high sequence similarity and virtually identical 3-dimensional structures, there are pronounced differences in sugar binding specificity. These differences in lectin activity and specificity are important because the toxicity and cytotoxicity of type 2 RIPs is (partly) determined by the binding of the B-chain to a sugar-containing receptor on the cell surface. Due to the extreme toxicity of ricin and abrin, type 2 RIPs are usually associated with highly toxic proteins (Barbieri, L., *et al*, 1993). However, type 2 RIPs show marked differences in (cyto) toxicity. Ricin, for example, causes 50% cell death at concentration below 1ng/ml, whereas some elderberry type 2 RIPs show no effect at mg/ml (Battelli, M. G., *et al*, 1997)

Type 3 RIPs are synthesized as inactive precursors (proRIPs) that require proteolytic processing events to occur between amino acids involved in formation of the active site (Mundy, J., *et al*, 1994). These RIPs are much less prevalent than type 1 or type 2 RIPs. To date, type 3 RIPs have been characterized only from maize and barley (Bass, H. W., *et al*, 1992; Chaudhry, B., *et al*, 1994; Reinbothe, S., *et al*, 1994).

1.4.2 ACTIVITY OF RIPs

Along with the effort to develop some RIPs into anticancer compound, attempts were made to find out what RIPs do and how they act. This led to the finding that RIPs

are RNA *N*-glycosidases that inactivate ribosomes through a site-specific deadenylation of the large ribosomal RNA (Endo *et al*, 1987; Endo, 1987; Endo, Y., 1988).

Once it turned out that the so-called single chain protein synthesis inhibitors share a substantial sequence similarity with the A-chain of ricin, the first functional link between type 1 and type 2 RIPs becomes obvious and the search for a common working mechanism started. This search soon revealed that ricin, abrin and PAP inhibit cell-free protein synthesis by irreversibly inactivating the ribosomes in such a way that the function of elongation factors EF-1 and EF-2 is blocked (Barbirie, L., *et al*, 1993; Sperti, S., *et al*, 1975). Ricin recognizes a highly conserved region in the large 28S rRNA and cleaves a specific N-C glycosidic bond between adenine and the nucleotide on the RNA whereby the adenine residue is removed. Due to the removal of this adenine, the deadenylated (or abasic) site becomes unstable and a β elimination reaction can occur after the RNA is treated with acidic aniline, whereby the 3' – end of the rRNA is cleaved and can be detected by electrophoresis. For the most often used substrate rat liver ribosome the specific site is A₄₃₂₄ in 28S rRNA. This site is usually depicted as being present in a single-stranded loop, called the sarcin / ricin loop. It is located in domain VII some 400 nucleotides from the 3' end of the RNA. Subsequent work revealed that this particular site-specific RNA *N*-glycosidase activity is a common property of all identified type 1 and type 2 RIPs.

Although all RIPs exhibit RNA *N*-glycosidase activity towards ribosomes, there are marked differences in substrate specificity. For example, ricin is highly active towards mammalian and yeast ribosomes but poorly active or even inactive on plant and

Escherichia coli ribosomes (Barbieri, L., *et al*, 1993). In contrast PAP depurinates ribosomes from plants, bacteria, yeasts, and lower and higher animals. Most type 1 RIPs have a rather broad specificity whereas type 2 RIPs have a preference for animal ribosomes. Both RIPs and ribosomes contribute to the apparent substrate specificity. Since the rRNA target structure is universally conserved, differences in sensitivity between ribosomes most likely reside within the ribosomal proteins, which may either allow or prevent access of the RIPs to the sarcin /ricin loop. Vater *et al.* identified rat liver ribosomal proteins L9 and L10e as the binding target of the ricin A-chain (Vater, C. A., *et al*, 1995), whereas yeast ribosomal protein L3 was identified as the binding factor of PAP (Hudak, K. A., *et al*, 1999). The specific interaction between PAP and L3 probably explains the broad spectrum activity of PAP towards ribosomes from species of different taxonomic groups because L3 is highly conserved in ribosomes. Differences in activity and ribosome substrate specificity are also due to differences in the structure of different RIPs. This was demonstrated by an approach in which PAP-ricin A-chain protein hybrids were created and examined for activity on rabbit reticulocyte and *E. coli* ribosomes. According to the results of these experiments, the amino-terminal half of the hybrid proteins determine the substrate activity. Structurally dissimilar surface polypeptide loops apparently do not play a role (Chaddock, J. A., *et al*, 1996).

The mechanism of entry onto the cells of protein toxins with intracellular sites of action has been of many studies in recent years (Olsnes, S., *et al*, 1985 and 1988). Nevertheless, it is not yet completely understood. The interest in toxin internalization is growing because this mechanism is a key point in a possible therapeutic utilization of

them or of their derivatives, and also because these studies give important insights in the intracellular transport and sorting of physiological ligands. In fact, much evidence currently suggests that toxin entry and routing inside cells are not toxin-specific but mimic pathways of physiological molecules.

RIPs can be taken up by cells through two mechanisms: the endocytosis dependent on the binding of RIPs to either the galactosyl residue (type 2 RIPs) or the mannose receptors on the cell membrane, and the fluid-phase endocytosis which allows the internalization of molecule without a receptor-mediated mechanism (Van Deurs, B., *et al*, 1989). The entry mechanism of type 1 RIPs is not well understood and only hypotheses can be formulated. Glycosyl residues are present in most of the type 1 RIPs, which could be internalized after binding to carbohydrate receptor on cell membrane. Peritoneal exudes cells (PEC) internalize gelonin by mannose receptors in a saturable manner. Mannan inhibites the specific binding and changes the pattern of gelonin uptake by PEC to the non-saturable pattern observed in cells lacking mannose receptors in spite of the gelonin to PEC (Madan, S., *et al*, 1992). The lower toxicity of the type 1 RIPs as compared to that of type 2 RIPs, suggests that the receptor-mediated internalization is not very efficient for type 1 RIPs. However, a considerable amount of evidence suggests that type 2 RIPs do not cross directly the plasma membrane but enter the cytosol by endocytic pathway. Electron microscopic studies show that a ricin-ferritin conjugate clusters first at the cell surface and appears in the endocytic vessels 60 minutes latter (Nicolson, G.L., 1974). Endocytosis is temperature dependent process. Thus, very little intoxication can be observed if cells are exposed to RIPs at 0 °C and

then washed with the competing ligand before restoring a physiological temperature (Sandvig, K., *et al*, 1979). Metabolic inhibitors protect the cells from toxin activity by preventing endocytosis, which is an energy consuming process (Sandvig, K., *et al*, 1982). The time lag of 30 minutes observed between exposition to ricin and inhibition of protein synthesis correlates with the time required for the intracytoplasmic visualization of the toxin linked to horseradish peroxidase (Gonatas, J., *et al*, 1980). Therefore, endocytosis appears to be a necessary step in the internalization of RIPs. Gelonin, and possibly other type of RIPs, could be internalized by the fluid-phase endocytosis into cells lacking the receptors for the glycosyl residues present in the RIP molecule (Madan, S., *et al*, 1992).

Covalently cross-linked RIPs to specific antibodies or cytokines (immunotoxins) have successfully been used in cancer immunotherapy. Immunotoxins, combines the respective benefits of the toxicity and specificity of monoclonal or polyclonal antibodies which recognize antigenic determinants on the cell surface of the tumor cells as vehicle for delivery of toxic agents (Lambert, J. M., *et al*, 1988; Singh, V., *et al*, 1989 and 1992; Laemmeli, U.K., 1969). Although immunotoxins were hailed as a new class of anticancer agent in the early 1980s, they have not measured up to their full potential (Singh, V., *et al*, 1992). Despite some bright spots, response rates generally have been low, and immunotoxins limited by their toxicity and narrow therapeutic window. The major disadvantage of immunotoxins is their toxicity to non target organs, which has limited the progress in the field of immunotoxin research.

Like antibodies, hormones are quite specific in their interaction with their receptors, therefore, analogous to immunotoxins, hormonotoxins (so called because the hormone component of the construct provided specificity for the toxin action) were constructed by conjugating gonadotropin hormones (such as oLH or hCG) with the RIP to target the hybrid complex to selective cells in the gonads (Carlsson, J., *et al*, 1978; Stripe, F., *et al*, 1980; Singh, V., *et al*, 1989; Srinivasan, Y., *et al*, 1985).

Physiological barriers hinder the effective delivery of drugs to tumors (Jain, R. K., 1998; Pluen, A. *et al*, 2001). To target cancer cells, a blood borne therapeutic agent must first cross the vasculature and then travel through the interstitium. Recent strategies have attempted to avoid these barriers by attacking the blood supply instead of cancer cells, either by suppressing the formation of new vessels (antiangiogenic therapy) or by abolishing established vascular network (antivascular therapy). This approach has the advantage that the delivery vehicle, once in the blood stream, should have direct access to the target endothelial cells. Recent studies have shown that cationic liposomes have a propensity for localizing in tumor vessels, but the mechanism behind this selectivity and the optimal formulation to maximize this effect have not been defined. Delivery of endogenous macromolecules to the cytosol is a fundamentally inefficient process. This difficulty arises from the fact that cells have an obligation to maintaining homeostasis; hence the need for strict control over what is allowed passage, into the cells. Because of their hydrophilicity and large hydrodynamic volumes, macromolecules such as DNA and protein are effectively impermeant to the cell's plasma membrane. Those that are taken by cells via, for example, fluid phase or

receptor mediated endocytosis are ultimately degraded within the endosomal/lysosomal pathway, or in some cases are returned to the extracellular environment (Mellman, I., 1996; Mukherjee, S., *et al*, 1997). For many bio-molecules with therapeutic potential, direct interaction with an intracellular target may be a prerequisite for efficacy. This condition is particularly true for many plant-derived toxins that have cytostatic or cytotoxic activities and thus have potential as anti-cancer therapy. Gelonin, a 30 kDa type I toxin obtained from the seeds of *Gelonium multiflorum*, is an ideal candidate for therapeutic delivery due to its minimal toxicity when added extracellularly (Stripe, F., *et al*, 1980). Numerous strategies have been employed to efficiently deliver gelonin to the cytosol of cancer cells including conjugation of folate, hormones or monoclonal antibodies, and entrapment into liposomes or polymers (Alam, A., *et al*, 1992; Atkinson, S. F., *et al*, 2001; Betler, M. 1996; Lambert, J. M., *et al*, 1985; McIntosh, D. P., *et al*, 1982; Patrick, .N. G., *et al*, 2001; Rosenblum, M. G., *et al*, 1999; Singh, V., *et al*, 1989 and Veenendal, L. M., *et al*, 2002). These approaches are predominantly aimed at increasing cell-type specific binding and uptake and in some cases augmenting delivery into the cytosol.

1.5.0 LIPOSOMES AS TOXIN CARRIERS

Liposomes are versatile vehicles in terms of structural characteristics and mode of drug incorporation. This creates a wide range of options for the design of effective liposomal drug formulations to induce tumoricidal effector mechanisms.

Liposomes are phospholipids vesicles where lipids are arranged in a concentric bilayer enclosing an aqueous space. These structures form spontaneously when lipids are suspended in aqueous solution. Water soluble material can be accommodated in the aqueous space of liposomes, whereas lipophilic substances get associated in the lipid part. Size and charge of liposomes could be varied according to the requirement.

Liposomes are commonly prepared by ultrasonic irradiation of water dispersion of phosphatidyl choline (lecithin). However, this method suffer from the drawbacks that it provides a wide range of size heterogeneity and also results in degradation of phospholipids. If macromolecules like DNA, protein, enzyme etc, are present in the sonication mixture, these macromolecules undergo denaturation or degradation during sonication. To overcome these problems, quite a few alternative methods have been described (Bangham, A. D., *et al*, 1965; Batzri, S. and Korn, E. D., 1973; Deamer, D. and Bangham, A. D., 1976; Kagawa, Y. and Racker, E., 1971; Szoka, F. Jr. and Papahadjopoulos, D., 1978).

Phospholipids solution in organic solvent when injected in buffer gives rise to formation of liposomes. One of these methods which makes use of chloroform and methanol mixture as organic solvent was originally developed by Papahadjopoulos (Papahadjopoulos, D., 1978) has recently been modified to give large size unilamellar liposomes, which retain the entrapped protein intact (Alam, A., *et al*, 1992)). The method is simple and highly reproducible even at large scale preparation.

Structural versatility, innocuous nature, and easy biodegradability, in addition to other properties, have made liposomes a more potent biological carrier in drug delivery

and drug targeting. Most of its applications have been based on the ability of the liposomes to preferentially migrate to reticuloendothelial system (RES) or tissues such as liver and spleen (Vertut-Doi, *et al*, 1996). However, avoiding degradation of liposomes by mononuclear phagocytic system has been a problem (Vertut-Doi, *et al*, 1996). Nonetheless, in principle, liposome as drug carrier for targeted delivery has potentials of application in chemotherapy of cancer.

The successful application of liposomes as carrier for drugs and enzymes in therapy is heavily dependent on their stability in circulation, tissue distribution and also on their mode of interaction with target cell(s). Size and surface charge of the liposomes appear to control the rate of liposomes clearance from blood. Large liposomes are cleared more rapidly than smaller one (Juliano, R. L., *et al*, 1975; Hinkle, G. H., *et al*, 1978). Liposomal preparation of mixed sizes possesses biphasic rates of clearance, whereas liposomes of homogeneous size exhibit a simple exponential clearance (Gregoriadis, G., *et al*, 1974). The surface charge of liposome is also an important determinant of cellular clearance. Neutral and positively charged liposomes were cleared less rapidly than were unilamellar negatively charged one (Gregoriadis, G., *et al*, 1974). Survival of liposomes in circulation is also affected by the choice of its constituents. Incorporation of increased amount of cholesterol in liposomes promotes their stability in blood (Kirby, C., *et al*, 1980).

Liposomes have served dual role: as a valuable experimental tool for membrane research and in addition, as an *in vivo* delivery system for enhancing the efficacy of various biologically active molecules (Papahadjopoulos, D., 1978; Knight,

C.G., 1981; Ostro, M. J., 1987; Gregoriadis, G., 1988). Animal studies have shown that liposomes can decrease the toxicity of several anti-tumor and antifungal drugs (Rahman, A., *et al*, 1986; Forssen, E. A., *et al*, 1981; Olson, F., *et al*, 1982; Gabizon, A., *et al*, 1986; Lopez-Berestein, G., 1983). Moreover, liposomes have also been shown to be an efficient carriers for anti-parasitic drugs for treating intracellular infections such as Leshmaniasis (Black, E. D., *et al*, 1977; New, R. R., *et al*, 1978; Alving, C. R., *et al*, 1978), of macrophage activators for increasing tumoricidal activity in models of metastasis (Koss, W. C., *et al*, 1985), and of various antigens and adjuvants for enhancing the immune response (Alving, C. R., *et al*, 1990; Gregoriadis, G., 1989). These studies have led to several promising chemical trials (Sculier, J. P., *et al*, 1988; Gabizon, A., *et al*, 1989; Treat, J., *et al*, 1990; Lopez-Berestein, G., *et al*, 1985; Presant, C. A., *et al*, 1988).

Liposomes have been found not to accumulate appreciably in tissues such as heart, kidneys and gastrointestinal tract. This results in lowering of the toxicity of encapsulated agent to these tissues (Szoka, F. C., 1981). Major proportions of liposomes from circulation of injected animals are captured by liver and spleen (Gregoriadis, G., *et al*, 1972 and Gregoriadis, G., 1979). Other tissues such as lung, kidney, and skeletal muscle etc. uptake are rather modest and seldom exceed 2-5 % of the dose per gram of tissue (Gregoriadis, G., *et al*, 1974 and 1977).

Targeting of liposomes to specific cells in bio-phase does not guarantee the delivery of liposomal content to cells. The delivery is mainly controlled by the mode of interaction between liposomes and cells. Liposome-cell interaction may proceed by one

(or more) of the following modes: (a) stable adsorption, (b) lipid transfer, (c) fusion, and (d) endocytosis. Fusion and endocytosis are the two useful interactions which ensure delivery of liposomal contents to target cells (Pagano, R. E., *et al*, 1980).

Conventional liposomes are best studied for delivery to cells of reticuloendothelium system (RES), localizing their contents mostly in the liver and spleen within a minutes after intravenous administration. However, liposomes specifically targeted to tissues other than RES require the presence of special ligands on their surface for specific binding to cells or tissues, and in addition also require a long enough circulation time to be able to reach their target. These findings prompted us to investigate the cytotoxic effect of liposome encapsulated gelonin on liver cancer.

The present investigation is an attempt for the following objectives:

- ☞ Isolation and purification of gelonin from the seeds of *Gelonium multiflorum*.
- ☞ Determination of biological activity of purified gelonin.
- ☞ Induction of hepatocarcinogenesis in Swiss albino mice by intravenous administration of DBN.
- ☞ Encapsulation of gelonin into liposomes.
- ☞ Study the effect of liposome encapsulated gelonin on cancer regression by *in vitro* and *in vivo* experiments.