CHAPTER I

INTRODUCTION
Drugs are external chemical entity which are often required for the treatment of various diseases. Drugs are essential part of our way of life, right from the birth, we enter the world with the aid of drugs, to death where medicine helps us to minimal distress and even with a remnant of dignity. It is quite good to prevent diseases, but those who are unfortunate enough to contract the disease will be grateful for drugs.

Whenever a drug is given, a risk is generally taken. The risk is made up of the properties of the drug, of the patient and of the environment. There are several categories of chemotherapeutic agents like antimalarial, antitubercular and anticancer. These agents are often administered to palliate the disease which additionally encompasses severe toxicity. Of these, anticancer agents are quite toxic even at cellular level due to their cytotoxicity and are administered in a dreadful and invincible cancer. Therefore, during cancer chemotherapy, study of drug associated toxicity is required to be undertaken at histochemical and histopathological level to know as to how these drugs perform at this level, so that precise precautionary measures could be adopted.

Cancer is a disease whose existence was first recognized thousand of years ago. The term cancer which means ‘crab’ in Latin was coined by Hippocrates in the fifth century B.C. to describe disease in which particular tissues grow and spread unstrained, throughout the body, eventually choking off life. A ‘neoplasm’ as defined by Willis, is “an abnormal mass of tissue, the growth of which exceeds and is coordinated with that of the normal tissues and persists in the same excessive manner after the cessation of the stimuli, which evoked the change”.

2
Cancer is one of the most widely researched area as it has become the leading disease-related cause of death of the human population. It is the second leading cause of death since 1960s, behind cardiovascular disease. These are two main reasons for this change. First, cancer is a disease of multiple accumulating mutations that are becoming manifest in human population, which have enjoyed an increasingly prolonged life span. Secondly, cardiovascular related deaths are decreasing as a result of an increased understanding of the mechanism underlying the disease, the identification of risk factor and the development of targeted molecular therapies. In contrast, the medical treatment of cancer still has many unmet needs. The main curative therapies of cancer are surgery and radiation, which are successful only if the cancer is detected at an early localized stage. Once the disease has progressed to locally advanced cancer or metastatic cancer, these therapies are less successful [Jackson, 2000].

There are two main changes in a cancer cell [Gupta and Ganjir, 1998]

(i) One change is defined as being of a regulatory nature. The multiplication of the normal cells of an animal is carefully regulated; multiplication takes place only when it is required. The cancer cell, on the other hand, escapes the regulatory mechanism of the body and is envolved continuously in multiplication cycle.

(ii) Normal cells are confined to certain tissues, according to rule, on which the body’s overall architecture depends. The cancer cell is not confined to its original tissue but invades other tissue where it proliferates; i.e. metastasis.
About two hundred distinct types of cancer have been recognized. They can grouped into four main types: Carcinomas, Sarcomas, Lymphomas and Leukemias.

(i) ‘Carcinomas’ are tumors made up principally of epithelial cells of ectodermal and endodermal origin. The solid tumors in nervous tissues and in tissues of body surfaces, or their attached glands, are examples of carcinomas. They include cervical, breast, skin and brain carcinomas. About 85% of the cancers are carcinomas.

(ii) ‘Sarcomas’ are tumors made up principally of connective tissue cells, which are of mesodermal in origin. They are solid tumors growing from connective tissue, cartilage, bone and muscles. They constitute about the 2% of the human cancers.

(iii) ‘Lymphomas’ are cancers in which they is excessive production of lymphocytes by the lymph nodes and spleen. Hodgkin’s disease is an example of lymphoma. Lymphomas constitute about 5% of the human cancers.

(iv) ‘Leukemias’ are neoplastic growths of leucocytes (W.B.C.). They constitute about 4% of the human cancer.

Cancer is often perceived as a disease that strikes randomly and without warning. Most common cancers are caused by identifiable environmental factors, most prominently chemicals, radiation and viruses [Singhal and Arora, 1995].

According to Harvey [1980] the following are the principle mode to palliate the cancer:

i. Surgery
ii. Radiotherapy
iii. Endocrine therapy
iv. Chemotherapy
v. Immunotherapy.

Anticancer or cytotoxic drugs

Cytotoxic chemotherapy began with sulphur mustard which has been developed as chemical weapon in World War I [1914 -18]. Amongst their actions, depression and haemopoiesis were observed. Preparation of World War II [1939-45] included the research to increase the potency and toxicity of these odious substances. The disappearance of lymphocytes and granulocytes from the blood of rabbits was a useful marker of toxicity and gave rise to the idea of possible efficacy in lymphoid cancer. Nitrogen mustards, as an anticancer drug, were first tested on experimental lymphoma in mice and the results were sufficiently encouraging to warrant a therapeutic trial on human beings. The development of other classes of agents e.g. antimetabolites have been soon followed then after [Laurance and Bennet, 1992].

The success of cancer chemotherapy has been limited due to a lack of cell selectivity of cytotoxic drugs. They interfere not only with tumor cells but also with healthy cells. This leads to severe toxic effects and seriously limits the efficacy of cancer chemotherapy [Duve et al., 1974]

Chemotherapeutic intervention [Harvey, 1980]:

i. Phase specificity

Antineoplastic drugs are of two general categories (i) those that can act upon the cell throughout its cycle, such drugs are said to be phase - non
specific, (ii) those that can act preferentially during one or more of the non-resting phases; these drugs are said to be phase-specific. Phase specific drugs have greater activity during the growth phases. The particular phase during which a drug acts depends on the lethal mechanism.

(ii) Tumor selectivity and response

Those tumors with large growth fractions are more susceptible to chemotherapy than those with a low fraction. Examples of tumors that respond well to chemotherapy are acute leukemia in children, Hodgkin’s disease and breast cancer. Examples of neoplasm that respond poorly are malignant melanoma, carcinoma of the gastrointestinal tract, bronchogenic carcinoma and tumors of uterus and cervix.

Different cell types spend different proportions of time in one as opposed to another phase. Therefore, the most effective drug would be expected to be a type that is specific to the phase of longest duration. In part this may account for the differences in efficacy among drugs of different mechanisms and phase specificity.

(iii) Determinants of sensitivity and selectivity

In addition to the growth fraction of the tumor, other factors also determine the selectivity of drugs for certain cell type. The demand for nutrients also varies among tumor types but also differs between normal cells and tumor cells. Some drugs are metabolized in peripheral cell as well as in the liver. Different cell types differ in their ability to metabolize these drugs. Several drugs are converted to active metabolites by the target cells and differences in the rates of conversion may contribute to selectivity. Also
differences in penetrance account for some differences among drugs; lipid soluble drugs are more and effective than water soluble ones for neoplasm in the central nervous system.

(iv) Requirements for "kill"

A remission can usually be achieved with a kill of 90 to 99% of neoplastic cells. A kill of 99% would leave at least $10^7$ to $10^8$ surviving cells to carry on tumor growth and the remission would last only 3 to 4 doubling times. With those neoplasms against which the immune system is ineffective, a 100% kill is necessary to effect a true cure, since it has been shown experimentally that a single implanted neoplastic cell can develop into a tumor.

(iv) Combination chemotherapy

One way of increasing the percent kill is to combine two or more antineoplastic drugs.

(v) Log cell kill principle

Antineoplastic drugs may be characterized by their log cell kill index, that is, by negative log of fraction of the tumor cell population that survives a single course of treatment. Thus a drug that kills 99.99% of a tumor cell population, i.e. leaves 0.0001 (or $1/10^4$) of population, is known as 4 log drug; a second drug that kills 99.9% of tumor population is known as 3 log drug. The log cell index is a tenuous number, but it serves usefulness in predicting the effects of combination that meets criteria 1 and 2. A 4 log drug plus 3 log drug should provide 7 log drug combination that kills 99.99% or leaves $1/10^7$
of the population. A third drug that kills 99% (2 log drug) would further reduce the remaining population to $1/10^9$, which comes close to complete eradication of tumor.

**Classes of antineoplastic drugs**

Drugs currently used in chemotherapy of neoplastic diseases may be divided into several classes [Calabresi and Chabner, 1991]:

1. Alkylating agents
2. Antimetabolites
3. Antitumor agents of natural origin
4. Miscellaneous agents
5. Hormones and antagonists.

(i) **Alkylating agents**

These compounds are capable of introducing an alkyl group into another chemical molecule. They are highly reactive and interact with a variety of chemical structure. Most cytotoxic alkylating agents are bifunctional, i.e., they possess two reactive groups. Although these compounds can attack many chemical groupings in the cell but their major biological effect is believed to be due to their interaction with -

a. Nitrogen mustards: e.g. mechloretamine, cyclophosphamide, uracil mustard and chlorambucil.

b. Ethyl imines: e.g. triethylenendamine [TEM], thiotepa.

c. Alkyl sulfonates: e.g. Busulphan.
(ii) **Antimetabolites**

Antimetabolites are the substances with a molecular structure similar to that of a natural metabolite, which interfere with the function of the later. Farber and his colleagues introduced these compounds for the treatment of malignant growth. Antifolates occupy a special place in antineoplastic chemotherapy, in which they produce striking but temporary remission in leukemia [Farber et al., 1948] and is the first choice in case of solid tumors, chlorocarcinoma [Hertz, 1963]. There are several categories of antimetabolites:

a. Folic acid antagonists e.g. methotrexate
b. Purine antagonists e.g. 6-mercaptopurin
c. Pyrimidine antagonists e.g. fluorouracil.

(iii) **Antitumor agents of natural origin**

A number of naturally occurring substances have been observed to inhibit the growth of experimentally produced tumors in animals and some of them have been introduced into clinical practice. They are:

a. Vinca alkaloids e.g. vinblastin and vincristine
b. Actinomycin
c. Antibiotics : e.g. dactinomycin, daunorubicin and doxorubin.

(v) **Miscellaneous agents:** e.g. cisplatin, carboplatin, mitoxantrone, hydroxyurea and mitotane, etc.
(vi) Hormones and antagonists

Hormones have been used for the treatment of tumors arising from tissues such as mammary gland, prostate and uterus whose normal growth is dependent on hormones e.g. androgen, estrogen, progestines and corticosteroids. The normal prostate gland is suppressed by estrogens, apparently by a competitive antagonism of androgens, and estrogens are used to treat cancer of prostate gland, etc. Similarly, androgens exert an antiestrogen effect on certain breast tumors; only tumors of a cell type that contains estrogen receptors are responsive. Antiestrogens are also used to suppress such tumors.

Toxicity of anticancer drugs

Neoplastic cells have the composition and activities very much like those of the host cells. This is the basic problem in designing antineoplastic drug to make it possible to attack only on the tumor cells. At the same time it also imparts severe deleterious effects on normal cells. This, in turn, causes the toxicity in various organs.

PROFILE OF DRUG, SELECTED FOR PRESENT INVESTIGATION

Cyclophosphamide

Cyclophosphamide is an alkylating agent which has been of great interest for the past few years in cancer chemotherapy and in autoimmune diseases. It has also been widely used as an immunosuppressant during organ transplantation and conditioning for bone marrow grafting in combination with whole body irradiation [Thomas et al., 1973]. In addition to this, its
clinical use is compromised by the dose dependent/dose limiting toxicity to various organs [Connors, 1984].

**Structure and chemical name of cyclophosphamide.**

\[
\begin{align*}
\text{O} & \quad \text{N(\text{CH}_2\text{CH}_2\text{Cl})}_2 \cdot \text{H}_2\text{O} \\
\text{NH} & \quad \text{O}
\end{align*}
\]

2-[(Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine,2-oxide monohydrate.

**Molecular formula**  \( \text{C}_7\text{H}_15\text{Cl}_2\text{N}_2\text{O}_2\text{P} \cdot \text{H}_2\text{O} \)

**Molecular weight**  279.10

**Description**  White, crystalline powder, liquefies on loss of its water of crystallization

**Solubility**  Cyclophosphamide is soluble in water and alcohol.

**Clinical use and toxicity**

Cyclophosphamide is activated by hepatic enzymes [Livingstone and Carter, 1970]. After activation in liver, it is converted into phosphoramidine and acroleine which are active metabolites, having antitumor activity [Colvin et al., 1973]. The clinical spectrum of activity of cyclophosphamide is very broad. It is the drug of choice for the treatment of Burkitt’s tumor. It shares first choice for the treatment of chronic lymphocytic leukemia in children, multiple myeloma, squamous cell and large cell anaplastic carcinomas, adenocarcinomas of the lung and Ewing’s sarcoma. It is also effective on non-
Hodgkin’s lymphomas, breast tumor, ovarian and testicular tumors [Bonadonna and Valagursa, 1983]. It is also a class III immunosuppressive drug. Cyclophosphamide can completely prevent the development of autoimmune allergic encephalomyelitis in both rats and guinea pigs (Paterson, 1968). It has received considerable attention for the control of organ rejection after transplantation on non-neoplastic disorders. It has been shown to be of value in the treatment of rheumatoid arthritis, Wegner’s granulomatosis, idiopathic thrombocytopenic purpura, erythroid aplasia, childhood nephrotic syndrome, and dermatomyositis. It improves the survival of bone marrow and possibly of heart transplants.

Cyclophosphamide is quite toxic drug. Alopecia occurs in about 50% of the patients receiving maximal prolonged treatment. Leucopenia is the inevitable side effect. Other side effects include anorexia, nausea and vomiting, mucosal ulcerations, dizziness, occasional thromocytopenia, nail ridging, cutaneous pigmentation and occasional hepatic dysfunction [Harvey, 1980].

**DRUG DELIVERY SYSTEMS**

Enormous progress has been achieved in last two decades in the field of drug delivery systems which provide possible and new strategies for the administration of various bioactive agents by which targeting of the drug could be achieved in the organ of choice and simultaneously avoiding the accumulation of drugs in other parts of the body.

The conventional drug therapy has marginal role in the treatment of various chronic diseases. The archaic manner of introducing drugs to patient is
obviously inefficient and often associated with the toxic side effects. The encapsulation of pharmacologically active compound in microvesicular system offers desirable possibilities in future therapy. Encapsulated drugs protect the host from unwanted immunological effect and circulating vesicles may be acting as a slow release system, sustain drug levels in the blood at higher levels for longer periods than drug administered through orthodox systemic routes. Apart from this targeting of the drugs to a site of action such as tumors, a diseased organ could be achieved. The basic rationale for this concept is to achieve therapeutic response with minimal side effects.

**Erythrocytes as drug carrier**

Out of various delivery systems, biological approach seems to be attractive in which highly specific physiological recognition mechanisms are used as part of drug delivery approach. In this particular approach most commonly erythrocytes are used as biological carrier in which various bioactive agents can be incorporated to achieve controlled systemic levels coupled with targeting the site of action [Ihler, 1983].

There are several intriguing potential advantages in using erythrocytes as a drug delivery system [Ihler et al., 1970; Zimmerman et al., 1976]. The life span of erythrocyte is 120 days, and mainly function to transport and carry hemoglobin. Under microscope the red blood cells appear 7 to 8 μm in length on 2.4 μm in thickness. When erythrocytes are osmotically lysed, the pores on the membrane are opened and intra and extra cellular solutes takes place, therefore the drug present during lysis will be encapsulated and the pores are again brought to original state by maintaining its isotonicity. Most of the protein molecules associated with the red blood cell membrane are peripheral
membrane proteins associated with the cytoplasmic side of the lipid bilayer and held together with relatively weak electrostatic forces. The most abundant of these proteins is spectrin, which is long, thin and flexible rod about 100 nm in length that constitutes about 25% of the membrane associated protein mass (about $2.5 \times 10^5$ copies per cell). This spectrin extends its key role in maintaining the structural integrity and biconcave shape and behaves as a cytoskeleton of the cell. Spectrin is basically composed of two large polypeptide chains $\alpha$-spectrin ($\sim 40,000$ daltons) and $\beta$-spectrin ($\sim 220,000$ daltons). Each chain is thought to be made up of many $\alpha$-helical segments interwound in groups of three and connected by nonhelical regions. There are various desirable properties which make erythrocyte as a suitable drug carrier [Deloach et al., 1979].

i. They are natural product of the body and biodegradable.

ii. They are biocompatible.

iii. They can be easily prepared.

The basic advantages of using erythrocytes as carriers are [Deloach, 1986].

i. Selective targeting and attenuation of side effects.

ii. Provide sustained and controlled drug release.

iii. Wide varieties of drugs like protein and DNA molecule can be encapsulated.

iv. Targeting of biologically active compound can be achieved in reticuloendothelial system.
Application of erythrocytes as drug carrier

(A) Targeting potential

Targeting to reticuloendothelial system (RES) can be achieved through suitable surface modification (heat treatment, sulphydryl treatment, gluteraldehyde treatment, desialation, etc.).

(i) In treatment of Gaucher’s diseases

Glucocerebrosidase loaded erythrocytes are successfully used to decrease accumulation of glucocerebroside [Beutler et al., 1977].

(ii) In treatment of parasitic diseases

Eradication of parasites residing in RES can be achieved by targeting drugs encapsulated in erythrocyte to RES [Talwar and Jain 1992b].

(iii) For removal of RES iron load

Desferrioxamine loaded erythrocytes have been used to remove excess iron stored in a body [Green et al., 1980].

(iv) In treatment of lysosomal storage diseases

Lysosomal enzymes can be delivered to lysosomes of the erythrophagocytic cells and thus missing enzyme can be replaced [Thorpe et al., 1974].

(v) Targeting of mycotoxins to RES
To prolong the liver retention of T-2 toxin (tricothecene mycotoxins), erythrocytes encapsulated T-2 toxin has been used by Deloach et al. [1988].

**B. As circulating bioreactors**

These carrier system are extremely useful as circulating bioreactors, especially for enzymes. Entrapped enzymes may catalyze reaction to reduce the circulating levels.

**C. As circulating carriers**

Erythrocyte carriers can be used to disseminate bioactive agents for prolonged periods of time in circulation.

**D. In preventing thromboembolism**

Heparin encapsulated in erythrocytes, can be liberated from circulating carriers at the site of thrombus formation [Eichler et al., 1986].

**E. Targeting other than RES**

Erythrocytes can also be used as functional "processing centers" in the circulation and when urease is encapsulated it is extremely useful in the treatment of kidney failure [Rieman et al., 1975]. Apart from this, magnetically responsive erythrocyte carriers are reported [Jain and Vyas, 1994, Zimmerman et al., 1978].

Resealed erythrocytes with modified surface characteristics, gluteraldehyde treatment [Zocchi et al., 1991], antibodies [Pinilla et al., 1994], disialation and phenylhydrazine treatment [Mishra and Jain, 2000 b] are quickly removed from the circulation by phagocytic cells, located in liver
and spleen suggesting the possibility of using erythrocytes in targeting of bioactive agents.

The drug encapsulating erythrocytes have been used for the RES targeting in the treatment of lysosomal storage disease, liver tumors [Lewis and Alpar, 1984; Sprandel, 1994] and parasitic diseases [Talwar and Jain, 1992a].

Talwar and Jain [1992b] reported an increased hepatic concentration of unmetabolized primaquin by administering gluteraldehyde treated erythrocytes. Human erythrocytes were also evaluated as carrier for ciprofloxacin for prolonged antibiotic effect and selective localisation to the RES [Mishra et al., 1996 a].

Lot of work has already done regarding histochemical and histopathological effects induced by various pesticides, heavy metals and other chemicals, but reports of histochemical and histopathological changes due to carcinogenic drugs are very scanty. On the basis of literature survey it seems that no substantial work has been reported in this field. However, the contribution of some workers in this field is worth mentioning.

cyclophosphamide induced enhancement of experimental metastasis by splenectomy. Geetha et al. [1989a] mentioned doxorubicin induced alterations in glucose metabolism.


Aviles et al. [1999] reported cyclophosphamide associated uroepithelial toxicity. Mechanisms of resistance to the toxicity of cyclophosphamide [Games, 1999] were also investigated.

Ghosh et al. [1999] investigated the effect of ascorbic acid supplementation on liver and kidney toxicity in cyclophosphamide treated female
albino rats. Locatelli et al. [1999] compared the hepatic toxicity from cyclophosphamide, methotrexate and fluorouracil [CMF regimen].


There are plenty of reports available in which encapsulation of wide varieties of drugs, proteins and enzymes in erythrocytes have been observed. In these reports major thrust has been on behavior of these carrier system in blood plasma and passive vectoring in different organs which are quite often determined in terms of drug concentration in tissues.

In the field of present investigation, the literature is quite scanty, regarding histopathological aspects employing these novel carrier systems. Therefore, present research plan has been proposed so as to study the effect of cyclophosphamide, an anticancer drug, histopathologically in the vital organs of albino rats when introduced through drug delivery system, using erythrocytes as microvesicles.

**RESEARCH ENVISAGED**

Neoplastic cells have composition and activities similar to those of the host cells. This has made it far impossible to design anticancer drugs which will not attack normal cells. This, in turn, causes toxicity in various organs. This emphasizes the necessity to undertake the detailed toxicity of anti-cancer drug in various organs. The present study has been undertaken with a view to study various histochemical and histopathological changes in vital organs of albino rats used as a model animal.
Due to wide application in cancer chemotherapy, cyclophosphamide has been selected for the present investigation as an anticancer drug, to evaluate histochemical and histopathological change in vital organs viz. liver, lung, kidney and intestine at albino rat. The possible outcome of this study is likely to prove useful during the treatment of various types of cancers, in which precautionary measures could be taken by keeping in view the histochemical and histopathological alterations.

Apart from this, the conventional drug delivery system plays a marginal role in providing efficient treatment of neoplastic diseases. The archaic manner of administering drug is obviously inefficient and often results in severe toxic side effects. The encapsulation of pharmacologically active substances in microvesicles offers potential possibilities. Therefore, the present work has been undertaken keeping in mind the relevant toxicity of cyclophosphamide, through conventional drug delivery system and comparing with emerging novel drug delivery system, by encapsulating cyclophosphamide in erythrocytes. Encapsulated drug in circulating vesicles may by acting as a slow release systems providing sustained drug level in the blood at higher levels and for prolonged period.

**PLAN OF WORK**

1. Selection of anticancer drug and its characterization.
2. Determination of concentration associated toxicity on albino rats
   
   (i). LD$_{100}$, (ii). LD$_{50}$, (iii). LD$_{0}$
3. Histological studies of vital organs like –
   
4. Drug induced histochemical changes for example;

5. Qualitative comparison of histochemical changes in rats:
   (i). Control, (ii). Treated

6. Drug induced histopathological changes on various organs viz. Liver, Lung, Kidney and Intestine.

7. Comparison of histopathological changes in vital organs of rat after administration of:
   i. Free cyclophosphamide
   ii. Cyclophosphamide loaded gluteraldehyde treated erythrocytes (Acute study only).
   iii. Plain 0.9% NaCl solution (Control).

8. The toxicity will be studied in two phases:
   i. Acute study, and ii. Chronic study.