Chapter III

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3.1. HYPERTHERMIA AND CANCER

Cancer therapy is achieved by removal or killing of neoplastic cells. Radiation therapy (RT), chemotherapy (CT), surgery and hyperthermia (HT) are being used for management of cancer. The potential use of hyperthermia in cancer therapy is based on the rationale that elevated temperatures of 41 to 46°C inhibit proliferation of malignant cells.

3.2. BIOLOGICAL RATIONALE FOR USE OF HYPERTHERMIA IN CANCER THERAPY

Three major mechanisms have been proposed for the HT mediated cell killing.

(i) Heat affects cellular membranes by changing the permeability, composition or fluidity, ultimately leading to the death of the cell (Wallach et al., 1978 and Gerner et al., 1980). Heat effects on membrane fluidity have been implicated by the observed interaction of heat with membrane modifying drugs; both alcohols (Li et al., 1978) and local anaesthetics (Yatvin et al., 1977) have been shown to increase the thermal sensitivity of membranes. Several studies have shown similar relationship between membrane cholesterol levels and hyperthermic sensitivity (Cullis and Dekruijff, 1979; Lepock, 1983 and Grunner et al., 1985). Anderson et al. (1981) and Lepock (1982) observed a direct correlation between cholesterol-phospholipid ratio and adaptation of cells to growth at temperatures varying from 32 to 41°C. Much attention has been paid to the lipids of membranes and their influence on membrane fluidity in relation to cell killing (Yatvin et al., 1982). Bowler et al. (1973) observed an increase in membrane...
permeability after HT and a loss of membrane bound ATPase. These effects correlated with cell killing. These membrane changes also induce alteration in the ion permeabilities. An increased influx of calcium and efflux of potassium has frequently been observed (Leeper, 1985). Mitotic cells have been found to be very heat sensitive. HT treatment apparently prevents the aggregation of the globular proteins to the spindle apparatus or causes the disaggregation of spindles. In consequence mitotic cells are unable to complete the mitotic division and cells with a tetraploid genome enter into the G1 phase (Cress et al., 1982; Mitisuyuki and Hiraoka, 1990). In fast proliferating systems many tetraploid cells are seen (van Beuningen et al., 1978). In a similar way microtubules of the cytoskeleton disaggregate during HT treatment and reaggregate during incubation at 37°C (Lin et al., 1982). A correlation between cell killing and disturbances of the cytoskeleton has been observed, in dead cells no reassembly of the cytoskeleton occurs (Cress et al., 1982). In heavily heat damaged cells these structures have been completely lost. Hyperthermia affects "second messengers" such as Ca++ (Stevenson et al., 1984 and Calderwood et al., 1984; 1985), H+ (Hoffer and Mivechi, 1980; Yi et al., 1983 and Calderwood et al., 1985) and cyclic AMP (Calderwood et al., 1985).

(ii) Heat may kill cells as suggested in the histological observations of Overgaard et al. (1977) by an increase and damage in lysosomes of cellular cytoplasm. Disintegration of these lysosomal vesicle and damage by the released digestive enzymes might be the cause of cellular death. Lending support to this hypothesis
are reports of biochemical evidence of increased lysosomal enzyme activity in heated cells. However these studies determined enzyme activity following in vivo heat treatments and may reflect tissue response to physiological effects caused by heat, such as heat induced changes in blood flow, rather than direct cellular heat damage.

(iii) Another mechanism for heat killing involves thermal damage to proteins. Tomasovic et al. (1978) and Roti Roti et al. (1978) have reported an increased non-specific attachment of non-histone nuclear proteins to DNA following heat treatment; however, this phenomenon shows only limited correlation to heat killing and more likely is important in preventing repair of radiation damage (Dickson et al., 1980). Other investigations implicate heat effects on a number of protein functions such as DNA synthesis, RNA synthesis, protein synthesis and respiration (Dube et al., 1977; Tomasovic et al., 1978; Warocquier et al., 1969 and Mondovi et al., 1969).

3.3. BIOCHEMICAL ASPECTS OF HYPERTHERMIA IN CANCER TREATMENT

MICROENVIRONMENT AND THERMOSENSITIVITY

The micromilieu by which the cells are surrounded is very important for their thermosensitivity (Vaupel and Kallinowski, 1987 and Reinhold, 1985). This micromilieu is determined by two important factors namely Physiological and Metabolic (Fig.1).
FIG. 1

PHYSIOLOGICAL EFFECTS OF HYPERTERMIA

CONVOLUTE HEAT TRANSFER

INTERSTITIAL EDEMA

KETOCERESIS

LACTIC ACID PROD

TUMOR BLOOD FLOW

ACIDOSIS

ENERGETIC STATUS

TUMOR CELL DESTRUCTION

DIRECT EFFECTS

BIOCHEMICAL EFFECTS OF HYPERTERMIA

Glucose

(+)

Glut

ATP

(+)

NADH

NAD⁺

Lactate

↑↑ (pH ↓↓)

Pyruvate

NAD

Lactate

↓

Acetyl-CoA

↓

Citrate Cycle

CO₂

FADH₂

NADH

OXPHOS

(-)

ATP ↓ (-)

(+) stimulation

(-) inhibition

BIOLOGICAL EFFECTS OF HYPERTERMIA
Physiological Factors

Effects of HT on tumor microvasculature: During hyperthermia, the blood pressure on the arterial side is low, while on the venous side the intravascular pressure is high. This results in decrease of the arterial venous pressure gradient, and therefore there is decrease in erythrocyte velocity in the capillaries. In these conditions the sheer rate of blood flow decreases, and it has been shown that under these circumstances, in combination with a low pH, the flexibility of the erythrocytes in the capillaries decreases. This enhances the ongoing vascular stasis mechanism (Jain, 1988 and Schmidt-Schonbein et al. (1984). Similarly HT mediated arterial venous shunting was observed by Dewhirst et al. (1984). The development of vascular stasis in tumor has been suggested by Lee et al. (1986). They observed accumulation of fibrin in the tumors during hyperthermic. Further work carried out by Copley (1980) showed that under these conditions of low pH occurring close to the wall of capillaries in an acidified tumor fibrin is converted to fibrinogenin (a gel like material) directly. This mechanism is considered as one of the factors contributing to the development of vascular stasis during HT. In addition, aggregation of erythrocytes, leukocyte sticking, thrombosis and finally arteriolar vasoconstriction are observed (Strom, 1979; Endrich et al., 1979; 1984; 1986; Ackerman and Hechmer, 1978; Eddy, 1980; Badylak, 1985).

Another relevant mechanism leading to a drop in tumor blood flow during heat treatment may be the pronounced reactive hyperemia of the adjacent normal tissues surrounding the tumor. The vasodilation in the tissues in the neighbourhood of a tumor can lead to a steal phenomenon and thus diversion of blood from the tumor, which can
be so pronounced that a great portion of the tumor hypoperfusion during heating may be explained by this mechanism. The inhibition of blood flow at high thermal doses may lead to following changes in tumors (Fig. II):

(a) Can increase the cytoidal effect of HT.
(b) Can modulate pharmacodynamics of anticancer drugs via modification of the cellular microenvironment.
(c) Can further intensify the inefficient drug delivery in many tumors (changes in intra tumor pharmacokinetics).
(d) Can make tumors more hypoxic, thus reducing their overall radiosensitivity (Emami et al., 1981; Hammerson et al., 1985; Singh, 1990).

Impact of localized HT on the pH distribution in malignant tumors

Tumors in vivo convert large amount of glucose into non essential aminoacids and into the pentose component of nucleic acids. Besides glucose oxidation, cancer cells have increased anaerobic glycolysis resulting in accumulation of lactic acid. Due to the elevated rate of lactic acid production and its subsequent inadequate removal, a severe tissue acidosis is evident in malignant tumors (Coss et al., 1982 and Wike- Hooley et al., 1984).

Tissue acidosis can enhance the therapeutic efficacy of elevated tissue temperatures. This is due to the fact that:

(a) Lowered tumor pH values increase the cytoidal capacity.
(b) Low pH values inhibit the repair of thermal damage.
Tumor physiology and microcirculation during a hyperthermic treatment.
Tissue acidosis may inhibit the development of thermo-tolerance.

3.3.3. HYPERTHERMIA MEDIATED METABOLIC CHANGES

Hyperthermic treatment results in increased rates of metabolic reactions followed by dysregulation of cellular metabolism. These alterations have been studied especially in intermediary metabolism like glycolysis, citrate cycle, lipid metabolism, and oxidative phosphorylation. An increased turnover of ATP has been observed in cells and tissues during heating. These changes lead to a depletion of energy reservoirs. Also, dysregulation occurs at certain metabolic key points. Thus, the pathway of pyruvate into the citrate cycle via acetyl CoA is apparently reduced in heated cells. The redox ratios of lactate/pyruvate, NADH/NAD⁺ and others are decreased (Streffer, 1985).

Glucose and energy metabolism

Glucose metabolism plays a central role in designing novel strategies for modifying the thermosensitivity of cells. It is closely linked to the metabolism of lipids and some amino acids. Glucose is metabolized in all cells through the glycolytic pathway to pyruvate and under anoxic conditions to lactate. Under oxic conditions, pyruvate, oxidized counterpart of lactate, is metabolized to acetyl-coA, which is degraded in the citrate cycle. This pathway generates the reduced nucleotides which are needed as electron donors for oxidative phosphorylation. Thus, these metabolic pathways are linked to various factors, such as pH and oxygen tension. In some of the tumors, anaerobic glycolysis is the dominating pathway (Warburg et al., 1962). HT increase the
anaerobic glycolytic rate resulting in increased level of lactate in tumors (van Ardenne, 1980). Thus intracellular pH decreases leading to enhancement of cellular thermosensitivity. Several authors have demonstrated that ATP levels decrease during heating of cells in vitro (Francesconi and Mayer, 1979; Lunec and Cresswell, 1983; Jahde and Rajewsky, 1982; Mirtsh et al., 1984;). Thus an increased energy supply is needed during the heating of cells and tissues which is provided by an enhanced ATP synthesis, resulting in enhanced ATP turnover.

Inhibition of DNA, RNA and Protein synthesis

The initiation of DNA synthesis in new replicons and elongation of nascent DNA in these replicons and after HT treatment (Gerner et al., 1979; Henle and Leeper, 1979; Wong and Dewey, 1982; Warters and Roti, 1982). Heat causes an increase amount of single-stranded DNA. DNA elongation recovers faster than replicon initiation after a heat treatment (Warters and Stone, 1983). Thereafter the prolonged depression of heat induced DNA synthesis is apparently connected to the initiation processes. Warters and Stone (1983 a) have also reported that heat treatment caused a long term inhibition of ligation of replicative DNA fragments with a size as small as the length of a replicon after HT. The activity of the poly (ADP-ribose) synthetase decreases in human melanoma cells after heating to 42 to 44°C (Streffer et al., 1983; Tamulevicius et al., 1984). This enzyme is firmly bound to the chromatin and is involved in ligation of DNA fragments as well as in DNA repair processes (Shall, 1984). Enzymatic studies have shown that DNA polymerase B, which is involved in unscheduled DNA synthesis for
DNA repair, is more thermosensitive than the DNA polymerase (Dube et al., 1977). Simard and Bernhard (1967) based on electron microscopic observations have reported that heat treatment at 42°C destroys the structure of nucleoli which are the sites for the synthesis of rRNA in the cell nucleus. The processing of the 45S RNA, a precursor of rRNA, to the functional 18S rRNA is apparently blocked by heating (Warocquier and Scherrer, 1969; Ashburner and Bonner, 1979). After recovery from heat shock, synthesis of rRNA becomes quite heat resistant (Burdon, 1985). The RNA synthesis for certain proteins (heat shock proteins) is even enhanced (Overgaard and Wielson, 1984). In mammalian cells elevated temperatures lead to a breakdown of the active polypeptide synthesizing polysomes.

After cytotoxic heat doses an increased K efflux as well as enhanced Ca$^{2+}$ and H$^+$ influx have been reported (Ruifrok et al., 1984 and Anghileri et al., 1984). A dramatic activation of heat shock protein genes occurs after heat exposure in cells which develop thermotolerance (Gerweck and Bascomb, 1982; Finlay et al., 1988 and Ferrarini et al., 1992). These proteins have been found in many organisms (Schlesinger et al., 1982). Every investigated species synthesizes heat shock proteins (HSPs) in the molecular weight ranges of 60-74 kDa and 80-90 kDa (Burdon, 1985). A correlation of the synthesis of HSPs with the development of thermotolerance has been found in normal and malignant cells (Burden et al., 1982; Landry and Chretien, 1983; Tomasovic et al., 1983. Tomasovic et al., 1984; Schamhart et al., 1984; Omar and Lanks, 1984). Hahn, 1982 has discussed the role of ATP production through oxidative phosphorylation and mitochondrial membrane for cell killing by heat. A decrease in ATP level has been
observed in heated cells (Francesconi and Mayer, 1979; Ohyama and Yamada, 1980; Lunec and Cresswell, 1983; Mirtsch et al., 1984). However, at the same time the ATP turn over and hence also ATP synthesis are enhanced (Ohyama and Yamada, 1980; Streffer, 1985). With respect to the cell nucleus it is interesting that an increase of nuclear protein content occurs after hyperthermic treatment of cells (Roti Roti, 1982). This increase correlates with cell killing. The new proteins form a complex with the chromatin and many interfere with the formation of the DNA replication complex (Warters and Roti Roti, 1982).

**Glutathione**

Mitchell and Russo (1983) have postulated that the oxidative-reductive state may be very important for the cell’s response to thermal stress. They found that continuous heating of CHO cell at 42±1°C results in rapid elevation of cellular glutathione (GSH). GSH depletion by either diethylmaleate (DEM) or buthionine sulfoximine (BSO) before or during (42.5 or 43°C) hyperthermia results in thermal sensitization (Mitchell et al., 1983). However, once thermotolerance is induced, depletion of GSH has minimal effects on the subsequent heating response. Therefore cellular redox state (GSH levels) is important in the response of the cell to heat implying that oxidizing free radicals may be mediating heat damage.

**Polyamines**

Ben Hur et al. (1978) observed that exogenous polyamines sensitised cells to hyperthermia and inhibited thermotolerance development. Fuller and Gerner (1982)
proposed a mechanism of polyamine modulation of hyperthermia cytotoxicity. Heat induced leakiness may allow polyamines to escape into the extracellular medium where they interact with a target on the outer surface of the cell to potentiate thermal killing, suggesting that the heat induced release of compartmentalized polyamines and interaction with targets on the cell surface may be a normal component of heat cytotoxicity. Positively charged polyamines are capable of lowering the availability of certain membrane components such as phospholipids, cholesterol or membrane proteins (Schindler et al., 1980). Hyperthermia sensitization occurs at extracellular polyamine concentrations more than an order of magnitude lower than those observed intracellularly. This indicates that the plasma membrane is asymmetric in its sensitivity to polyamines during heating. The polyamine and polyhydroxyl modulation of the heat damage may be mediated through cytoskeletal alteration or through altering the cellular redox state.

DNA Replication

Inhibition of replicon initiation and elongation leads to single stranded regions of DNA, allowing exchanges to occur and chromosome aberrations to be produced that correlate with cell killing in the S phase. Procaine and low pH alter the effects of heat on DNA replication proportionally to their effects on survival (Davis et al., 1983; Warters and Stone, 1983a).
DNA Polymerase

The loss of DNA polymerase beta activity after heating correlates well with radiosensitization, but whether the loss of activity is due to alteration of chromatin structure or direct effect on the enzyme is not resolved. Since procaine affects polymerase beta activity only when whole cells are heated and not when the free enzyme is heated, and heating isolated nuclei is without effect compared to heating whole cells, there must be an important relationship between the plasma membrane, cytoskeletal elements, nuclear matrix and polymerase beta activity (Spiro et al., 1982; 1983 and Dewey and Esch, 1982).

Signal transduction

Hyperthermia causes a significant increase in the level of intracellular free calcium, an event which occurs rapidly after temperature elevation (Drummond et al., 1988; Calderwood et al., 1987 and Born et al., 1990). This probably in turn influences protein kinase C activity. Maher and Pasquale (1989) found that heat shock enhances tyrosine phosphorylation in many different cell lines. Activation of protein kinase and protein phosphorylation have been implicated as very important events in virtually all cell responses to external stimuli and in many instances these processes are Ca^{2+}-dependent, either through Ca^{2+}-calmodulin or Ca^{2+}-phospholipid dependent protein kinase C. PKC activation by hyperthermia is suggested by the observation that heat induces the accumulation of diacylglycerol, a natural activator of this enzyme. Activation of protein kinase C is involved in numerous cellular processes, including regulation of
cell growth, differentiation, gene expression and cellular metabolism. Protein kinase C is a phospholipid sensitive, Ca\(^{2+}\) ion dependent protein kinase. It plays an important role in the transmission of extracellular signals. If cells are stimulated by the attachment of biologically active substances to specific cell surface receptors, a receptor-coupled phospholipase C cleaves the membrane constituent phosphatidyl-inositol 4,5-bisphosphate yielding inositol triphosphate and diacylglycerol. The inositol triphosphate mediates the mobilization of calcium ion from intracellular storage sites (Berridge and Irvine, 1984) and diacylglycerol activates protein kinase C (Berridge, 1984).

Protein kinase C activity decreases both cytosol and particulate fraction. Hyperthermia causes a rapid and significant increase in the intracellular free Ca\(^{2+}\) and the increase is correlated with stimulation of phosphoinositide turnover (Drummond et al., 1988). PI activates PKC. Heat induced stimulation of PI turnover is transient, because prolonged heating causes depletion of PI as a result of which PKC also decreases. Heat treatment induces a change in subcellular distribution of PKC. This is because of translocation of enzyme from the cytosol to particulate fraction. The primary site for the action of hyperthermia seems to be the cell membrane. It is possible that PKC translocation and changes in membrane bilayer structure mediate some of the cell reactions (thermosensitivity, heat killing) to heat stress.
3.4. MECHANISM OF HYPERTERMIA MEDIATED CELL DEATH

The mode of cell death of tumor cells subjected to HT may be necrosis or apoptosis depending on the temperature and duration of heat treatment. Heating of tumor cells at mild hyperthermic temperatures (41-43°C) results in apoptosis while exposure to drastic temperatures (46°C) leads to cell death by necrosis (Takano et al., 1991).

Apoptosis and cancer: The additional effect is dysregulation of apoptosis. Apoptosis or programmed cell death is an active process of cell suicide characterized by the distinct morphological features such as condensation of chromatin, cell shrinkage, and formation of nuclear and cytoplasmic membrane-bound fragments called apoptotic bodies. The apoptotic bodies are phagocytosed by macrophages or neighbouring parenchymal cells.

At the molecular level, the signal mechanisms mediating apoptosis are not completely understood though expression of c-myc, c-jun and c-fos oncogenes has been implicated. The triggering of apoptosis leads to the activation of a Ca^{2+}-Mg^{2+} endonuclease resulting in DNA fragmentation. Tumor suppressor gene p53 may act as an inducer/mediator of apoptosis (Wx and Levine, 1994) while oncogene bcl-2 is a suppressor of apoptosis (Marx, 1993). Failure of apoptosis in tumor cells may be of fundamental importance in contributing to the resistance both to natural defenses and clinical therapy. The understanding of these phenomena will help in designing novel strategies for treatment of cancer.
3.5. CLINICAL HYPERTHERMIA

On perfusion of liver in rats and rabbits, an increased plasma half life of doxorubicin was observed due to reduced biliary excretion (Mimnaugh et al., 1978, Skibba, 1982) at temperatures 41-42.5°C. Systemic heat treatment (41°C for 45 min) caused higher plasma and tumor melphalan concentrations than in unheated animals, but the effect was greater in plasma than in tumor (Honess et al., 1985). In dogs, systemic HT at 42°C significantly increased clearance, volume of distribution and T 1/2 of free ultra filtrable cisplatin and the parent drug (Riviere et al., 1986). In man no change in half life of total cisplatin was seen on infusing the drug during whole body HT (Gerad et al., 1983), but enhanced nephrotoxicity was observed. It is known that the cytotoxicity of several antitumor drugs is enhanced by hyperthermia. Emi et al. (1992) used Sarcoma 180 (S-180) tumors and examined the effects of 5-flurouracil (5-Fu) and combined oral preparation of 1-(2-tetrahydrofuryl)-5-fluorouracil (FT) and uracil in a molar ratio of 1:4 (UFT), in combination with HT. The antitumor effect of 5-FU was not enhanced significantly by HT. Growth inhibition by UFT plus HT was significantly greater than that by UFT alone, whereas inhibition by UFT alone was significantly greater than that by 5-FU. The intracellular metabolism of 5-FU and FT in whole homogenate of S-180 cells, human tumor cell lines (SC-2 and LU-99), and five fresh human tumor tissues also was investigated. Conversion of FT to 5-FU, phosphorylation, and degradation of 5-FU were assayed with [3H] FT or [3H] 5-FU, and the products separated by thin layer chromatography. The conversion of FT to 5-FU and the phosphorylation of 5-FU were more rapid at 43°C than at 37°C, whereas the degradation of 5-FU to 2-fluro-β-alanine.
remained unchanged. This acceleration of the active metabolism of FT and 5-FU may be one explanation for the enhanced effect of UFT by HT.

3.6. MANIPULATIONS TO ENHANCE HEAT DAMAGE

A number of strategies are being tried for enhancement of biological damage produced by given exposure to heat (i) the use of glucose to reduce pH in tumor cells and consequently increase heat damage. (ii) Hydralazine, a vasodilator that causes increased blood flow in normal tissues thereby diverting blood flow from tumor, leading to an increase in heating and heat damage. (iii) step-down heating leads to greater increase of the thermal enhancement ratio (TER) in tumors than in normal tissues.

3.7. HYPERTHERMIA AS COMBINED MODALITY

In clinical practice various combinations of HT, CT and or RT are being tried for therapy of different cancers.

HYPERTHERMIA AND RADIATION

HT shows greatest clinical promise when it is used in combination with RT or CT. A combination of HT and RT is of potential benefit in cancer therapy. Tumor cells in G and M phases of the cell cycle are sensitive to radiation while cells in S phase are radioresistant. HT kills these radioresistant S phase cells, hence a combination of HT and RT is more lethal to tumor than either modality applied alone (Mitsuyuki and Hiraoka, 1990; Suit and Grewack, 1979). The intratumor hypoxic regions that are acidic and
nutrient deficient are more sensitive to heat damage. In addition HT also shows a synergistic action when combined with RT (Adams, 1984). It acts as a radiosensitizer by inhibiting repair of sublethal and lethal DNA damage caused by RT. It does so not by inactivating repair enzymes but by modifying the structure of DNA so that RT damage is masked. It has been reported that low oxygen pressure increases the radioresistance of cells (Alper, 1979) and due to the lesser density of blood vessels in tumors than in normal tissues the oxygen pressure is frequently lower in tumors than in normal tissues. Therefore the hypoxic cells in tumors increase the radioresistance of these tumors and the occurrence of such cells is an important cause for the failure of radiotherapy in many cases (Hall, 1978 and Dewhrist et al., 1984; Streffer, 1985; Hartson-Eaton and Hornback, 1986).

**HYPERTERMIA AND CHEMOTHERAPY**

HT enhances the cell killing potential of some anticancer drugs (Magin, 1980; Engelhardt, 1986; Fidler, 1986; Hoffer, 1987 and Dahl, 1988). The biochemical rationale of HT induced enhanced chemosensitivity are: (i) HT mediated damage to the plasma membrane resulting in increased cell permeability of drugs, e.g., adriamycin; (ii) inhibition of repair of DNA damage caused by drugs, e.g., bleomycin, (iii) temperature dependent increase in drug action such as alkylation. Hahn et al. (1977, 1979); Ganapathi and Grabowski (1983); Coleman (1988) have shown the interaction of heat with a variety of cytotoxic agents. The type of drugs, dose, temperature and time of administration of agents are important factors in determining cell kill by combination
of these agents (Hahn et al., 1982 and Marmor, 1979).

Three types of drug interactions have been categorized (Hahn et al., 1979) [Table A]. The first are drugs which show an increase in effectiveness with increased temperature below 42°C. Examples of these drugs would be several of the alkylating agents, such as nitrosoureas (Field et al., 1979 and Oliverio, 1973). The second category of drugs show increased effectiveness only above a threshold temperature value such as bleomycin (Braun et al., 1975 and Olson et al., 1982) shows a threshold for infraction with 43°C. The third category are those drugs which are normally not considered of any value therapeutically at 37°C but which show significant killing ability at elevated temperatures. Amphotericin B, a polyene antibiotic, would fit this category. Agents fitting in category one would be effective for use in whole body heating where the temperatures cannot exceed 42°C. The second category of drugs would be useful for local hyperthermia where heat treatments are expected to exceed 43°C. While many drugs show increased effectiveness when combined with hyperthermia, the great variety in mechanisms of drug killing preclude the idea that heat and drug interactions are simple unilateral phenomena. In fact, instances have shown that heat can enhance drug resistance in the case of adriamycin (Harisiadis et al., 1975) and actinomycin D (Donaldson, 1978). As heating duration is increased, cells in culture become highly resistant to killing by either drug. This may be due to heat induced alteration in drug transport into the cell (Field et al., 1979; Eksborg and Ehrsson, 1985; Herman et al., 1988).
Table A

**HYPERTHERMIA AND INTERACTION WITH DRUGS**

1. Drugs which show no temperature threshold effects

   Thiothepa
   Nitrosourea group: BCNU
   CCNU
   Cisplatinum
   (probably suitable for systemic hyperthermia)

2. Drugs which show a temperature threshold effect, around 43°C, before a major potentiation is observed:

   Bleomycin
   Adriamycin
   Actinomycin D
   (Probably suitable for local hyperthermia)

3. Drugs which are not normally cytotoxic at 37°C but cause considerable cell kill above a threshold temperature:

   Amphotericin-B
   Cysteamine
   AET (2-amino-ethyl-isothiourea)
   Cysteine
3.8. ANTICANCER DRUG MELPHALAN

Alkylating agents: Nitrogen Mustards.

(i) Mechlorethamine

(ii) Cyclophosphamide

(iii) Chlorambucil

(iv) Melphalan (Alkeran, L-PAM, L-Phenylalanine mustard).

Melphalan is an amino acid derivative of mechlorethamine and was synthesized with the hope that some tumor cells might have specific transport systems for phenylalanine. Malignant melanoma cells utilize this amino acid as a precursor for melanin synthesis (Teicher et al., 1981, Fig III).

Mechanism of action

Melphalan does not require metabolic activation. However, the substituted phenyl ring greatly reduces the reactivity of the molecule in solution by slowing the rate of ionization of the chlorides and cyclization of the ethylimino groups. Melphalan when placed in aqueous solution loses a chloride ion and forms a cyclic ethylenimonium ion. This carbonium ion interacts with nucleophilic groups, such as the N⁷ and O⁶ of guanine, and leads to an interstrand cross-linking of DNA. These intermediates also interfere with the activity of enzymes, co-enzymes and other substances that regulate cellular activity.
The initial intramolecular cyclization of chloroethyl group of Nitrogen mustards, produces a highly electrophilic ethylenimonium ion, which can react with various nucleophilic compounds such as guanine in the N7 position. Subsequent cyclization of the other chloroethyl group may lead to cross-linking of macromolecules.
3.9. TARGETED DRUG DELIVERY

The therapeutic efficacy of anticancer drugs administered orally or intravenously is restricted by dose limiting toxicities against normal tissues in vivo. In addition, some drugs are metabolized rapidly and their period of effectiveness is relatively short. Hence the drugs can be targeted to specific tissues by means of drug carriers (targeted drug delivery systems) like microdispersions, microdroplets, microcrystals and liposomes.

Modes of Targeted Drug Delivery

Some of the approaches currently used to improve the therapeutic index of anticancer drugs by targeting of drugs specifically to tumor tissues are by the following modalities:

Microdispersion

Microdispersion technology permits the formulation of water insoluble compounds as uniformly dispersed suspensions of spherical microparticles having uniform diameters. These formulations are suitable for a number of routes of administration, including intravenous & oral. Characteristics of this particles includes (1) Submicrometer size they can be sterile filtered (2) Extremely narrow distribution eliminating the necessity for post-preparation processing such as mechanical milling (3) Excellent in vitro suspension & in vivo stability-providing long shelf-life & no particulate aggregation with blood components (4) Complete drug composition eliminating loading problems when carriers are required.
Water insoluble antineoplastic agents have been formulated as particulate suspension suitable for intravenous administration. Early results from in vivo tests have already shown enhanced efficacy & reduced adverse effects with this formulation compared with water soluble formulations.

Microdispersion overcomes the problem of drug hydrolytic instability because the compound is susceptible to hydrolysis only after it has been solubilized from the particulate form after intravenous administration.

Microdispersion technology creates some of the smallest delivery particles when compared with other drug delivery methods, including microencapsulation of insoluble drugs, (Nozawa et al., 1981) incorporation of drugs in microspheres, (Widder et al., 1980) formulation of lipophilic drugs in emulsions (El-Sayed et al., 1983) and incorporation of drugs in liposomes (Gabizon et al., 1983 and Abra et al., 1983 and Weinstein et al., 1984).

Microdroplet and microcrystal technologies

Microdroplet technology involves incorporation of a drug into microscopic droplet of a biocompatible organic liquid phase, which are delivered in fine submicrometer-sized aqueous suspensions. The drug-containing microdroplets are encapsulated and stabilized against coalescence by a layer of natural phospholipids.

The microcrystal technology is an improved and simplified version of the microdroplet formulation technology. One of the alternative strategies being tried for increasing the therapeutic efficacy of anticancer drugs is encapsulation of drugs in lipid vesicles or liposomes.
3.9.1. LIPOSOMES

Liposomes are artificially generated lipid vesicles which entrap drugs within their aqueous compartments and/or lipid bilayer membranes (Bangham et al., 1974). These vesicles have been regarded as promising drug delivery systems for the treatment of cancer because encapsulation of drugs in liposomes results in increase in their antitumor effects and decrease in their side effects on normal tissues (Gabizon et al., 1983; Papahadjopoulos et al., 1976 and Weinstein et al., 1984 (Fig IV).

Classification of liposomes

Apart from their lipid composition, which determines properties such as membrane fluidity, charge density and permeability, liposomes can be characterized by their size and shape. They are classified as:

(i) Multilamellar Vesicles (MLVs)

Multilamellar vesicles cover a wide range of sizes (100-1000nm), each vesicle consisting of 5 or more lamellae. Vesicles consisting of just a few concentric lamellae are called oligo or paucilamellar vesicles. These arise spontaneously when bilayer forming lipids are hydrated in aqueous solution.

(ii) Small Unilamellar Vesicles (SUVs)

Small unilamellar vesicles range from 25-100nm in size and contain only a single bilayer membrane enclosing an aqueous compartment. These vesicles are
LIPOSOME (MLV)

- Water soluble molecules
- Lipid soluble molecules
- Water soluble molecules with hydrophobic moiety penetrating lipid phase

Neutral, negative, or positive charge
formed by ultrasonic, electro capillary emulsification, or solvent dilution techniques.

(iii) Large Unilamellar Vesicles (LUVs)

These liposomes have a diameter of the order of 100 - 500nm, contain a single bilayer membrane formed by reverse emulsion, and are very efficient in entrapping water soluble drugs (Deamer and Uster, 1983; New, 1990). A number of techniques have been used for the preparation liposomes each having its own advantages and disadvantages. These are:

(a) **Sonication:** It is used for the preparation of SUVs (Huang, 1969).

(b) **French Press:** It is used for the preparation of SUVs (Barenholtz et al., 1980).

(c) **Ethanol Injection Method:** It is used for the preparation of SUVs (Batzri and Korn, 1987).

(d) **Ether Injection Method:** This procedure is used for the preparation of LUVs (Deamer, 1978).

(e) **Reverse Phase Evaporation Method:** It is used for the preparation of LUVs (Szoka and Papahadjopoulos, 1978).

(f) **Detergent Dialysis:** It is widely used when proteins are to be reconstituted into the liposomal membrane (New, 1990).

(g) **Dehydration-Rehydration Method:** It is used for oligo-lamellar, multilamellar and unilamellar vesicles (Kirby and Gregoriadis, 1984).
TDD SYSTEM USING LIPOSOMES

Targeting of liposomes to tumor tissues can be achieved by physical or chemical methods. The physical properties of liposomes such as pH, phase transition temperature, photo-sensitivity and magnetic properties can be used for TDD (Brenner, 1989; Gregoriadis, 1975 and Newmann, 1986). Alternatively chemical properties viz., antibodies, hormones, lectins etc. may be exploited for targeting liposomes to tumor tissues.

Mechanism of interaction of liposomes with cells

Interaction of a liposome and a cell can take several forms. A liposome can adsorb to almost any type of cell. An adsorbed liposome may be endocytosed, or internalized, by certain kinds of cells, may exchange lipid with cell membrane or may fuse with it. Fig(V) demonstrates the various modes of uptake of liposomes by cells (a). After adsorbing to a cell a liposome is likely to release its contents, some of which may enter the cell, depending on the nature of the contents and the type of the cell involved. (b) An endocytosed liposome is processed by a lysosome, an intracellular digestive organelle, after which the lipid components of the liposome may be incorporated into the cellular membranes, whereas the aqueous solutes that escape lysosomal degradation may be incorporated into the cytoplasm (c). A liposome that undergoes lipid exchange takes up lipids of the cell membrane and in return gives some lipids to the cell membrane (d). When a liposome fuses with a cell, the liposomal membrane merges with the cell membrane and the liposomes cargo becomes part of the cells cytoplasm.
Fig. V.

- A. Adsorbed liposome
- Aqueous solute
- B. Liposome undergoing endocytosis
- Undigested particles excreted
- Lysosome
- Lipids excreted by lysosome
- D. Fused liposome
- C. Liposome undergoing lipid exchange

Liposome - Cell Interaction
3.9.2. LIPOSOMES IN CANCER THERAPY

Liposomal doxorubicin

A number of investigators have evaluated the utility of liposome encapsulation of doxorubicin hydrochloric acid (HCl) as a means to ameliorate toxicities while maintaining or improving the antitumor effect of the drug (Forssen et al., 1979; 1981; 1983; Gabizon et al., 1982; 1983; 1986; 1991; 1991a; Mayhew et al., 1992; Doidon et al., 1986; Storm et al., 1987 and Treat et al., 1990). Using liposomes of various compositions and sizes, animal studies from a number of laboratories showed that the acute and chronic toxicities of doxorubicin HCl can be attenuated while antitumor potency and efficacy are maintained or improved.

The mechanisms for improved therapeutic index of liposomal doxorubicin HCl as compared with free drug are as follows:

1. Sites of toxicity such as gastrointestinal tract are less susceptible to drug exposure by using liposomal drug formulations because the continuous capillaries present in GIT do not allow liposomes to escape the circulation, or the organ parenchymal cells are not capable of incorporating liposomes by endocytosis. This would result in decreased drug at toxicity sites and an improved therapeutic index if drug remains available to destroy tumor cells.

2. Reticuloendothelial cells, particularly those found in the liver and spleen, may take up liposomes containing doxorubicin HCl and act as reservoir for slow release of drug. Recent studies have shown that toxicity, including chronic cardiotoxicity, can be reduced by continuous intravenous infusion of doxorubicin.
After decades of research, liposomes encapsulated doxorubicin entered clinical trials during the past 5 years (Wallach, 1977; Cowens et al., 1990; Rahman, 1990; 1990a and Batist et al., 1991; 1992). In a phase II study in metastatic breast cancer, 24 patients with disease in liver, lung, lymph nodes, skin & bone were treated at 75 mg/m² of liposomal doxorubicin (Rahman, 1990). There were 9 out of 17 major responses (1 complete response and 8 partial responses) that occurred in visceral and soft tissue sites of disease. Nausea, when present, was moderate; mucositis was uncommon with only 2 out of 17 episodes of grade 3 toxicity observed. Grade 3 or 4 neutropenia was common; 13 out of 17 patients required dose modification because of febrile neutropenia. There was no severe platelet toxicity. The highest cumulative doses of liposomal doxorubicin administered to date were 1,275,750,675 and 485 mg/m². No patient experienced clinical cardiotoxicity. Trials to test doxorubicin efficacy in other types of cancer, in which its usage has been limited primarily because of toxicity, have been planned, and the outlook for the expanded use of this drug is more optimistic (Rahman et al., 1985; 1986 and Janoff et al., 1992).

Liposomal platinum

Perez-soler et al. (1987; 1988; 1989) and Khokkar et al. (1988) developed lipophilic cis-diaminocyclohexane-di chloro platinum derivates for liposome entrapment to be used for the treatment of tumors that involve the organs of the reticuloendothelial system and/or that are resistant to cis-platin. A liposomal formulation of one of these compounds
(cis-neodecanoato-trans R,R-1,2-diamino cyclohexane platinum (II); NDDP) was selected for further development. In preclinical toxicology, liposome-entrapped NDDP (L-NDDP) was found to be devoid of nephrotoxicity. The major toxicity in mice was myelosuppression and in dogs it was an acute and diffuse hemorrhage syndrome involving the gastrointestinal tract primarily. L-NDDP was found to be active against tumor models resistant to cis-platin & more active than cis-platin against liver and spleen metastases of M5076 reticulosarcoma and RAW 117 H-10 lymphoma in mice.

Liposomal vincristine

A liposomal vincristine-preparation has been produced that exhibits reduced toxicity as well as antitumor potency in murine L1210, P388 and B126 tumor models, when compared directly with free vincristine. Because of the altered biodistribution and the inability of liposomes to cross blood-nerve barriers, it is postulated that the neurotoxicity of vincristine will be decreased by liposome encapsulation.

Magnetic microcapsules

To achieve targeted distribution of anticancer drugs and sustained activity ferromagnetic ethylcellulose micro capsules containing the anticancer drug mitomycin (FM-MMC-mc) were prepared by a method based on phase separation principles. Animal studies showed that the release of drug from magnetic microcapsules could be magnetically controlled in the artery and urinary bladder. Vx2 tumor in the rabbit hind limb and urinary bladder were successfully treated with magnetic controlled release of
FM-MM-mc. A pharmacokinetic study revealed that the targeting of the microcapsules markedly enhanced the drug absorption into the surrounding tissues for a prolonged period of time (Kato et al., 1984).

Targeting Chemotherapy for hepatoma

Lipoidol ultrafluid has been used as a carrier for anticancer agents (oily anticancer agents). This arterial infection therapy was performed on 323 patients with hepatoma. Serum alpha fetoprotein (AFP) levels decreased in 93% of 177 AFP-positive patients. Reduced tumor size was observed in 210 of 212 assessable patients with unresectable hepatoma. The 1-, 2-, 3-, and 5-years survival rates were 84, 47, 37 and 34% respectively (Konno, 1992). A dramatic improvement in therapeutic index in human for any of these drug delivery system is yet to be proven.

3.9.3. TEMPERATURE SENSITIVE LIPOSOMES

Phosphatidyl choline membranes: Phase transitions

At different temperatures, lecithin membranes can exist in different phases, and transitions from one phase to another can be detected by physical techniques as the temperature is increased. The most consistently observed phase transitions is the one occurring at the highest temperature, in which the membrane passes from a tightly ordered "gel" or "solid" phase, to a liquid-crystal phase at raised temperatures where the free movement of individual molecules is higher. Membranes made from egg yolk lecithin show phase transition from -15°C to -7°C, compared with membranes from
mammalian sources which are usually in the range of zero to 40°C.

Phase transitions and fluidity of the phospholipid membrane are important both in the preparation and use of the liposomes, since the phase behaviour of a liposome membrane determines such properties as permeability, fusion, aggregation and protein binding, all of which can markedly affect the stability of liposomes and their behaviour in biological systems (Lee, 1977).

Yatvin and his coworkers (1978) have used a unique alternative approach for controlled release of drugs by using temperature sensitive liposomes in combination with local hyperthermia. They encapsulated hydrophilic agents such as methotrexate and cis-Dichlorodiammineplatinum into either small unilamellar vesicles composed of dipalmitoyl phosphatidyl choline (DPPC) and distearoyl phosphotidyl choline (DSPC) or reverse phase evaporation vesicles composed of DPPC and dipalmitoyl phosphatidyl glycerol (DPPG). Tomita et al. (1989) reported the use of amphiphilic and lipophilic agents for entrappment in temperature sensitive liposomes.

Local HT might increase the therapeutic efficacy of drug containing liposomes in the treatment of neoplasm by:

(i) promoting selective drug release at temperatures near the liquid phase transition of the liposomes;

(ii) increasing local blood flow;

(iii) by increasing endothelial permeability to particles, thereby enhancing accumulation of liposomes in the target tissues;
Schematic view of the release of drug from temperature sensitive liposomes in tumor heated to the phase-transition temperature.
(iv) increasing the permeability or susceptibility of target cells to the drug released from the liposomes;
(v) increasing direct transfer of drug from vesicles to cells, for eg., by fusion or endocytosis (Laudonio et al., 1990) FIG. VI

3.9.4. GENE TARGETING

The use of liposomes as delivery agent for DNA and other polynucleotides is a natural extension of their earlier application as drug delivery agents (Smith et al., 1993). Liposomes have many advantages as delivery agents. By altering their formulations or incorporating temperature or pH sensitivity, these vesicles may be targeted to specific tissues, or designed to mimic the properties of specific cell types eg, liposomes can be made to reproduce the native component of red blood cells which shield them from the attention of macrophages. The aqueous internal volume of liposomes is extremely advantageous as it allows the introduction of compounds that may also alter the metabolic state of the cells as the foreign DNA is introduced. This is a largely unexplored means of influencing gene delivery and expression.

3.9.5. LIPOSOMES AS CARRIERS OF ADJUVANTS:

Liposomal adjuvants are most effective when included in the liposomes that contain the immunogen. However, complex and poorly defined interactions of protein immunomodulators such as IL-2 with liposomes may occur on rare occasions which can cause unpredictable non-specific enhancement of immune reactions to liposomal
antigens. A variety of lipid adjuvants and protein mediators can influence the immune response to liposomal antigens. The most commonly used adjuvants for practical immunization procedures are endotoxins (including lipid A and lipopolysaccharide) and many lipophilic derivatives of muramyl dipeptide (Alving, 1991). When encapsulated in liposomes muramyl dipeptide (MDP) enhanced the immunogenicity of liposomally associated ovalbumin and induced macrophage nonspecific tumoricidal activity better than free MDP (Sone and Fidler., 1980). The incorporation of polysaccharide as coating containing MDP were more cytotoxic compared to MDP encapsulated in liposomes with out polysaccharide. The enhanced activity of liposomal MDP or liposomal MDP derivatives could be due to protection from extracellular inhibition or degradation.

Studies have shown that liposomes can serve as effective vehicles for inducing humoral immunity to a wide range of liposomal antigens (Alving, 1987; van Rooijen and Su, 1989). Some investigators have found that apparently non-immunogenic antigens with Freund’s complete adjuvant or with aluminium hydroxide become highly immunogenic when prepared with appropriate liposome formulation (Alving, 1991). Investigators have reported that with certain antigens or under certain conditions liposomes can be stronger adjuvants that complete Freund’s adjuvant. Some investigators emulsified liposomes with complete Freund’s adjuvant in a bid to induce an enhanced immune response to the liposomal antigen (Latif and Bacchawat, 1987). It has been shown that liposomes can induce antibodies having conformation specificities. Das et al. (1982; 1982a) reported the production of antibodies against mono saccharides such as D-mannose, galactose and N-acetylglucosamine. Latif and Bachhawat (1984) observed that liposomes carrying
galactose bound to their surface induced and immune response to entrapped antigen that was comparable to that of sugar-free neutral liposomes, where as, mannose coupled liposomes evoked a response that was equal to that of free antigen without complete Freund's adjuvant. These observations suggest that mannose-bearing liposomes are rapidly taken up and cleared by macrophages rendering them less immunogenic.

Numerous studies have shown that liposomes act as immunologic adjuvants for a wide spectrum of incorporated bacterial and viral antigens relevant to human and veterinary immunization. These include diptheria (Allison and Gregoriadis, 1974) and tetanus toxoid (Davis et al., 1986), Streptococcus pneumonia serotype 3, hepatitis B surface antigens (Manesis et al., 1979), and derived polypeptides (Sanchez et al., 1980), Epstein Barr virus antigen (Epstein et al., 1985), foot and mouth disease synthetic peptides (Francis et al., 1985), Plasmodium yoelii. Michalek et al., 1984; Pierce and Sacci, 1984; Pierce et al. (1985) have shown that lipid A or a uridine could act as an adjuvant for a local IgA response to a mucosally applied antigen, when either of them and the antigen are associated on a liposomal carrier.

3.9.6. IMMUNOLIPOSOMES

Targeting of liposomes with monoclonal antibodies directed against the target cell has been attempted by many investigators. Leserman et al., 1980 reported that liposomes bearing monoclonal antibodies against β-microglobulin bound specifically to human cells and not mouse cells. They further demonstrated that liposomes conjugated with monoclonal antibodies against the major histocompatibility complex of murine H-2
haplotype could selectively inhibit radiolabeled deoxyuridine incorporation by spleen cells from CBA (H-2) mice but not C57Bl/6 (H) mice. Furthermore Bragman et al. (1983) clearly showed that liposomes covalently coupled with the monoclonal antibody against human glycophorin selectively bound to K562 cells, a human leukemic cell line with glycophorin A. Based on the results of these targeting studies, Hashimoto et al. (1983) successfully treated a murine mammary carcinoma with monoclonal antibody-conjugated liposomes containing actinomycin D. Many workers have raised monoclonal antibodies against human AFP, and conjugated it to liposomes containing adriamycin. The therapeutic effects of these liposomes were investigated in vivo on AFP-positive human hepatoma strain maintained in nude mice. The first injectable liposomal vaccine trial in human was initiated in oct 1989. The vaccine consisted of liposomes that contained recombinant protein having epitopes derived from the repeat sequences of the circumsporozoite protein of the human malaria parasite, Plasmodium falciparum. The vaccine was found to be safe and induced high levels of antibodies (Alving and Richards, 1990; Brynestad et al., 1990; Alving, 1991).

3.9.7. IMMUNODIAGNOSTICS

Liposomes have recently been tried a tool for immunodiagnostics. The most current application in this field is membrane lytic assays. The method is based on trapping a fluorescent or enzyme probe inside liposomes; upon binding of an antibody with an antigen these liposomes become leaky. Using purified antibody or antigen a standard curve can be established; the concentration of unknown sample of antigen or antibody
can then be determined from the curve. The main advantages of this assay over traditional ones are: (a) Calorimetric and fluorimetric measurements can be used thus avoiding the use of radio isotopes; (b) a single lytic event generates a high degree of signal amplification due to the release of many signal molecules, and (c) it is not necessary to separate free antibody or antigen from immune complex and thus assay is homogeneous. The major disadvantage is that some serum samples contain a non-specific factor(s) which cause damage to liposome and ultimate release of the marker. This assay popularly known as "liposome immune lysis assay" or LILA to study the interaction of antibodies to lipid antigens including "Forsman glycolipid". The "LILA" assay system has been exploited to detect the patients with systemic lupus erythematosus (SLE), hemolytic anemia, Behcet’s disease etc. Yasuda et al. (1982) used a modification of this assay to measure urinary "microalbumin". Thus LILA shows promise in immunodiagnostics because of its simplicity, sensitivity, low cost and safety.

The work of Allen and Chonn (1987) and Forssen (1988) open new areas of investigation and potential clinical application. One may design the liposome to target reticuloendothelial system. This is the aim for treatment of Leishmaniasis caused by the Leishmania parasites, which replicate in the macrophage (Nacy et al., 1989); for therapy for liver pathogens, eg., Mycobacterium avium or Candida (Lopez-Berestein et al., 1985; Sculier et al., 1988 and Gangadharan et al., 1989) and for achievement of immune stimulation, eg., delivery of muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) to macrophage (Fidler et al., 1980). Alternatively one may blockade the reticuloendothelial system to sequential liposome injections (Proffitt et al., 1983) or design
a liposome to avoid macrophage uptake, leading to longer systemic circulation time with reduced hepatosplenic uptake and tumor tissue uptake (Allen et al., 1987 and Gabizon et al., 1988; 1989). Attachment of monoclonal antibodies to the liposomal membrane may further improve targeting. Focusing on liposomal drug encapsulation as a method of improving tumor drug delivery technique. Other promising applications are being explored. Reduction of rapid deamination by encapsulating cytarabine or a derivative that requires enzymatic activation could enhance the therapeutic efficacy of this important antileukemic drug (Rubas et al., 1986). Liposome alone have immunoadjuvant properties (Gregoriadis et al., 1989) that can be exploited by use as immunostimulants or as carriers for antigens relevant to human and veterinary immunization. Empty liposomes with a radioactive label (Ga or In) find use as tumor imaging agents (Proffitt et al., 1983; Gabizon et al., 1988).

Exploitation of liposomal uptake by macrophages or hepatocytes has potential for delivery of immunostimulators or antibiotics in the treatment of infectious diseases, particularly those associated with severe immunosuppression, such as leukemias, lymphomas and acquired immunodeficiency syndrome. The rate and extent of localization of liposomes in the RES depends on the surface of the liposomes. Negatively charged liposomes are cleared from the circulation at a faster rate than neutral liposomes and their uptake by the liver and spleen is largely in comparison to neutral liposomes (Gregoriadis, 1973; Gregoriadis and Neerunj, 1974; Gabizon and Papahadjopoulos, 1992). On the other hand, positively charged liposomes are eliminated at a slower rate and the uptake by the liver is found to be lower
(Gregoriadis, 1973a; Gregoriadis and Neerunjum, 1974; Jonah et al., 1975). Some of the authors have reported that negatively and positively charged liposomes activate the complement system which leads to their removal from circulation.

3.9.8. STEALTH LIPOSOMES

To avoid excessive liposome uptake by the phagocyte rich organs and thus to prolong the vesicles lifetime in blood, diverse 'stabilised' vesicles have been introduced. The long lived liposomes were first described by Allen and colleagues (1983). This formulation contained a special glycolipid, ganglioside GM1, as the decisive, uptake-suppressing liposome component. Further, their improvement and appreciable cost reduction was achieved by the invention of second generation of long lived liposomes which contained polyoxyethylene derivatives of phosphatidylethanolamine as the longevity mediating ingredients. Several patents have also been applied for in this field. The clearance rate of liposomes from blood (Juliano and Stamp, 1976; Allen and Everest, 1983) as well as their accumulation in tumors (Gabizon et al., 1990) depends on particle size in addition to specific lipids (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Gabizon et al., 1990). Accumulation of small neutral liposomes (DSPC/Chol) in tumors had been observed earlier (Proffitt et al., 1983). Several workers have enhanced this accumulation by longer blood half life and increased their usefulness as drug carriers by using vesicles having 100nm diameter. The high recovery in blood by the use of PEG liposomes and their accumulation in tumors may prove to be of considerable advantage to future clinical applications for the following reasons:
The PEG derivative is synthetic phospholipid easily prepared at high purity in larger quantities and considerable lower cost compared to the GM₁ ganglioside, which is derived from bovine brain.

Fluid and solid bilayer compositions give similar blood circulation times, adding considerably to the flexibility needed for various clinically relevant formulations.

The additional presence of the negatively charged PEG in the bilayer allows for better encapsulation of a variety of positively charged drugs and other macromolecules and reduce the likelihood of aggregation.

Dose independence dictates that a constant percentage of the injected dose is taken up by various tissues, irrespective of the amount injected, allowing for the reliable prediction of drug levels during therapy (Bosworth and Hunt, 1982; Allen and Hansen, 1991). Finally,

Reduced uptake by RES enhances the chances for localization in the other tissues for therapeutic and imaging implications.

Many investigators have studied liposomes as a carrier for the delivery of therapeutic or diagnostic agents. However, clinical applications of these liposomes have been limited by a rapid uptake into reticuloendothelial system (RES). The role of charge bilayer rigidity and size of liposomes has been examined to increase the uptake of liposomes into tissues other than liver and spleen (Abra, 1980 and Allen, 1987). Allen and Chonn (1987) reported that the sphingomylein which had a bilayer rigidifying effect showed an increase in circulation time and a concomitant decrease in uptake into
RES compared with phosphatidyl choline/Ch liposomes. Furthermore some attempts have been made to reduce the liver uptake of radioactive liposomes by pretreatment with a high dose of unlabelled liposomes (Kao, 1981) and with reticuloendothelial blockades such as dextran sulphate (Souhami et al., 1981; Patel, 1983). Another important approach to suppress the uptake of liposomes by RES and target these vesicles towards specific tissues is to incorporate glycolipids into liposomes because glycolipids present on the cell surface are considered to play an important role in various biological recognition processes. Liposomes having β-galactoside on their surface were preferentially taken up by parenchymal cells, whereas liposomes having α-mannoside were taken up by non-parenchymal cells (Ghosh, 1980; 1982) and liposomes containing mannosylated phospholipids were targeted selectively to macrophages. It was found that liposomes containing sialoganglioside, especially, ganglioside GM₁, showed a reduced uptake by the RES, resulting in prolonged circulation times (Gabizon, 1988; Allen, 1989).

Souhami et al. (1981) observed larger changes in clearance of liposomes from sheep red blood cells in mice whose RES was blocked with dextran sulphate. Blocking may not affect all the cells of RES equally, or different mechanisms of clearance may exist for liposomes and larger particles such as colloids or cells. It has been hypothesized that alternations in capillary permeability or vascular structure, which have been observed in animals (Underwood et al., 1972 and human tumors allow the smallest liposomes to pass out of the circulation and into the tumor. After RES blocking, the loaded neutral vesicles would remain in circulation longer and thus have a higher probability of diffusing through the permeable tumor capillaries (Proffitt et al., 1983). The targeting of
liposome encapsulated compounds to murine tumors may have significance tumor
diagnosis and therapy. If human tumors accumulate liposomes to a similar extent, then
labelled vesicles might be useful in determining tumor response to therapy, locating
metastasis, or even detecting, previously undiagnosed tumors. Finally, since many
anticancer drugs have been loaded into liposomes treatment of tumors that do accumulate

\(^{111}\)In-labelled vesicles might be made more effective by subsequently administering
vesicles containing an appropriate antineoplastic drugs. Another approach to decrease the
uptake of liposomes into RES is to use a different injection method from i.v. injection.
Ellens et al. (1981) reported that liposomes composed of sphingomyelin and
cholesterol (3:2 molar ratio) after intraperitoneal injection (i.p.) were transported from
intact peritoneal cavity to blood and their uptake by liver and spleen was reduced to
1/2-1/3 compared to i.v injection. Allen et al. (1989a) also reported that circulation levels
of liposomes formed from sphingomyelin, PC and ganglioside GM, after i.p. injection
were similar to that observed by i.v. injection, but that liver and spleen uptake was much
lower at 2hrs following i.p. injection. Parker et al. (1981) and Hisaoka et al. (1982)
showed that i.p. injection of the liposomes enhanced their uptake by lymph nodes.

3.9.10. TARGETED DRUG DELIVERY SYSTEMS FOR CIRCUMVENTION
OF MULTI DRUG RESISTANCE

The emergence of drug resistant tumor cells remain a major problem in cancer
chemotherapy. Drug resistance is manifested in two major forms: intrinsic or innate drug
resistance, which relates to the failure of many tumors to respond to initial
chemotherapy, and acquired drug resistance or multidrug resistance which occurs when a tumor initially responds to chemotherapy but later relapses and shows a broad resistance to the original drugs as well as to a variety of unrelated drugs. The phenomenon of MDR has been extensively studied using mouse or Chinese hamster cells (Kessel et al., 1968; Biedler et al., 1970; Dano et al., 1973) as well as human cells (Beck et al., 1974; Tsuruo et al., 1983; Bhalla et al., 1985; Akiyama et al., 1985; Shen et al., 1986). Isolation of tissue culture cells with a broad spectrum of cross resistance to many natural products anticancer drugs showed reduced accumulation of drugs in these cells due to the over expression of an energy dependent drug transport protein, P-glycoprotein (P-gp), pump. Rahman et al. (1992) reported that liposome-encapsulated doxorubicin modulates the multidrug resistance phenotype in Chinese hamster LZ cells (Thierry et al., 1989) as well as human colon cancer cells (Oudard et al., 1991). Further they concluded that liposome encapsulated doxorubicin, with its inherent MDR-reversing capacity, proved to be potentially effective modality for the treatment of human cancers. Circumvention of MDR by liposome encapsulated doxorubicin was confirmed by Perez-Soler et al. (1988) and Fan et al. (1990).