5. Design of Liposomes for Hyperthermia Mediated Drug Release
Introduction

The efficacy of liposomes as target drug delivery systems in cancer chemotherapy has been reported and several types of liposomal formulations have been explored, such as those bearing carbohydrate determinants (lectins), monoclonal antibodies, glycoproteins and antigens (Gregoriadis and Ryman 1972; Gregoriadis and Neerunjun 1975; Juliano and Stamp 1976; Gregoriadis 1988). The therapeutic efficacy of many of these formulations has however been limited by things such as antigenicity of the ligands, inability of the drug to reach the appropriate cellular compartment in active form and the presence of the endothelial or other histological barrier between the liposomes and its cellular binding site. In an attempt to resolve some of the problems it has been suggested that liposomes could be combined with local hyperthermia to achieve preferential release of drug in a targeted area (Yatvin et al., 1978; Weinstein et al., 1980; Tacker and Anderson 1982; Iga et al., 1991). The drug containing liposomes are formed using a phospholipid mixture that exhibits a gel state to liquid phase transition ($T_g$) at temperatures few degrees above the physiological temperature, the range easily obtainable by local hyperthermia. It is postulated that such liposomes would remain reasonably stable in the vascular system at normal body temperature ($T<T_g$) and, when passing through the heated area, they would release their contents when the transition temperature ($T_g$) is attained. This approach of using thermoresponsive liposomes has been tested using various antitumour drugs in combination with local hyperthermia of the tumour and the results obtained were indicative of the preferential accumulation of the drug in heated tumours (Weinstein et al., 1980; Yatvin et al., 1981; Bassett et al., 1986).

The present study was undertaken to explore the feasibility of employing thermosensitive liposomes of bleomycin and plumbagin in combination with hyperthermia as a bimodality approach for management of melanoma B16F1 in mice.

*Part of the work presented in this chapter has been published: see below*


Bleomycin

Bleomycin is a mixture of basic cytotoxic glycopeptides produced by the growth of *Streptomyces verticillus* or by other means (Barrows, 1995). Its main components are bleomycin A₂ and bleomycin B₂. Its structure is shown below;

![Bleomycin structure](image)

\[
\text{C}_{55}\text{H}_{84}\text{N}_{17}\text{O}_{21}\text{S}_3, \text{H}_2\text{SO}_4 \quad \text{(Bleomycin A}_2\text{ sulphate)}
\]

\[
\text{Molecular weight: 1516.62}
\]

\[
\text{C}_{55}\text{H}_{84}\text{N}_{20}\text{O}_{21}\text{S}_2, \text{H}_2\text{SO}_4 \quad \text{(Bleomycin B}_2\text{ Sulphate)}
\]

\[
\text{Molecular weight: 1523.63}
\]

Bleomycin acts as a cytotoxic agent by causing fragmentation of DNA. It also inhibits incorporation of thymidine into DNA (Barrows, 1995). It stops the progression of cells through the G2 and M phase of the cell cycle. In spite of these actions, it has very limited effect on bone marrow, a circumstance that gives it a special usefulness in drug combinations or combinations with other treatment modalities such as hyperthermia. It is useful in the treatment of squamous cell carcinomas of head, neck, oesophagus, skin and genitourinary tract, including the cervix, vulva, scrotum and penis and in treatment of Hodgkin’s disease, Kaposi’s sarcoma and warts. It is also used in the management of melanoma in combination with other antitumour agents (Barrows, 1995). Pulmonary toxicity is the major limiting factor in the use of bleomycin as a chemotherapeutic agent. There are reports of reduced toxicity and improved antitumour activity of bleomycin after encapsulation in vesicular carriers such as niosomes (Raja Naresh et al., 1996b,c)
Plumbagin

Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) is a crystalline substance present in *Plumbago rosea*, *P. europaea*, *P. zylanica* and *P. indica*. The structure of plumbagin is shown below:

Plumbagin (C_{11}H_{10}O_3). Molecular weight: 188.18

Plumbagin is prescribed for cancer treatment in the Siddha system of medicine (Uma Devi et al., 1994). It has also been reported to act against chemically induced fibrosarcoma in mice and against P388 leukemia *In vitro*. It acts like a mitotic inhibitor in arresting cell division (Singh et al., 1996). The antimitotic activity of the drug is expressed by occurrence of mitotic anomalies such as polypoidy, micronucleus, anaphase bridges, giant cells, stickiness and jaggling of the chromosomes. The antimitotic activity is also revealed by the reduction of mitotic index value. Plumbagin showed regression of experimental animal tumours (Singh et al., 1996). It is highly toxic drug and acts like a spindle poison by inhibiting cell mitosis at low concentrations and at higher concentrations it exhibits radiomimetic, nucleotoxic and cytotoxic effects. Wide variation in LD50 has been reported but the general agreement is that plumbagin is a highly toxic compound with a narrow margin of safety. Encapsulation of plumbagin in particulate carriers such as niosomes and microspheres reduced the toxicity of the drug and improved antitumour activity in variety of tumour cell lines including melanoma B16F1 in mice (Raja Naresh et al., 1996a; Singh et al., 1996; Kini et al., 1997; Oomen et al, 1999)

The anticancer activity of the above chemotherapeutic agents i.e. bleomycin and plumbagin can be increased and toxicity reduced by selectively targeting these drugs to tumour cells using novel thermosensitive liposomes in combination with localised hyperthermia. In the present study, we report some pilot experiments examining the utility
of thermosensitive liposomes in combination with localised hyperthermia for the management of melanoma B16F1 in mice.

Materials and Methods

Bleomycin used was a commercially available lyophilised powder for injection (Bleocin®, Khandelwal Laboratories Ltd., Mumbai, India). Plumbagin, Dipalmitoyl phosphatidylcholine (DPPC), Distearoyl phosphatidylcholine (DSPC) and Sephadex G-50 were procured from Sigma Chemicals Co., St. Louis, MO., USA. The purity of DPPC and DSPC was assessed by TLC (Stationary phase; Silica gel G, mobile phase; chloroform/methanol/water; 64:24:4, visualisation with I₂ vapor). All other chemicals and reagents were of analytical grade.

Standard plot of bleomycin

Standard plot of bleomycin was prepared in isotonic phosphate buffered saline (pH 7.4). Stock solution of bleomycin (one mg per mL) was prepared in respective buffers. Aliquots of the stock solution were serially diluted with the same buffer solution to obtain 1, 5, 10, 20, 30, 40 and 50 µg/mL of bleomycin solution. Absorbance of the final solutions was measured at 289 nm (UV absorption maxima of bleomycin). The standard plot of bleomycin in isotonic phosphate buffer saline (pH 7.4) is shown in Fig. 41.

Standard plot of plumbagin

Standard plot of plumbagin was prepared in isotonic phosphate buffered saline (pH 7.4) containing 30% PEG-400. Stock solution of plumbagin (one mg per mL) was prepared in buffer. Aliquots of the stock solution were serially diluted with the same buffer solution to obtain 1, 2, 4, 6, 8, and 10 µg/mL of plumbagin solution. Absorbance of the final solutions was measured at 269 nm (UV absorption maxima of plumbagin). The standard plot of plumbagin in isotonic phosphate buffer saline (pH 7.4) containing 30% PEG-400 is shown in Fig. 43.
Fig. 42. Standard plot of bleomycin in isotonic phosphate buffer saline (pH 7.4)

\[ y = 0.0102x + 0.0022 \]
\[ R^2 = 0.9986 \]

Fig. 43. Standard plot of plumbagin in isotonic phosphate buffer saline (pH 7.4) containing 30% PEG-400.

\[ y = 0.0683x + 0.0022 \]
\[ R^2 = 0.9986 \]
Preparation of liposomes

For bleomycin large unilamellar liposomes (LUV) and for plumbagin small unilamellar liposomes (SUV) were prepared as follows.

Thermosensitive large unilamellar vesicles (LUV) of bleomycin were prepared using reverse phase evaporation method as reported by Szoka and Papahadjopoulos (1978). To get a lipid composition with the phase transition temperature near hyperthermia temperature, a mixture of DPPC and DSPC was used. Briefly, DPPC (221 µM) and DSPC (23 µM) (9:1 w/w) were dissolved in 30 mL of isopropyl ether-chloroform mixture (1:1 v/v). The lipid solution was mixed with 5 ml of phosphate buffered saline (1/10 dilution, pH 7.4) containing drug in a 100 mL round bottom flask using a mixer (Remi Instruments, Mumbai). The obtained w/o emulsion was homogenised with a Vibra Cell Sonicator, W-375 (Sonics and Materials Inc. Co. Danbury, USA) and the organic solvent in the emulsion was evaporated gradually by a rotary evaporator at 60°C to form a liposomal suspension.

The entrapment efficiency of the liposomal formulation was determined by separating the unentrapped drug from liposomes by gel filtration on Sephadex G-50 column. The column was prepared using 1.5 g of Sephadex G-50 powder kept in double distilled water for 48 h for swelling. One ml of prepared liposomal suspension was placed on the top of the column and elution was carried out using phosphate buffered saline. Liposome entrapped drug eluted at the void volume of the column, whereas non-entrapped drug was retained and eluted later. The absorbance of the unentrapped drug was measured spectrophotometrically at 289 nm. The amount of drug still remaining within the vesicles was further confirmed by treating a known quantity of the separated liposomes with 1% v/v Triton X-100 and sonicating briefly to disrupt the vesicles. The resulting mixture was centrifuged and the supernatant assayed for drug content spectrophotometrically.

Thermosensitive small unilamellar vesicles (SUV) of plumbagin were prepared by thin film hydration and subsequent sonication (Weinstein et al., 1979). Briefly, DPPC and DSPC (9:1 w/w) were dissolved in chloroform: methanol mixture (9:1 V/V) (180 mg lipid per 9.5 mL of solvent) along with a known quantity of drug. The solvent was evaporated.
using a rotary flash evaporator under reduced pressure. Phosphate buffered saline (pH 7.4, 5 mL) was warmed to 60°C and then added to the flask with gentle agitation. The mixture was intermittently mixed on a vortex mixer. The resulting suspension was then sonicated under nitrogen at 50 °C for 1 h with the microtip of a Vibra Cell sonicator, W-375 (Sonics and Materials Inc. Co. Danbury, USA) at a nominal output of 20 watts. The sonicate was rapidly cooled in an ice bath, and any titanium fragments released from the microtip during sonication were removed by centrifugation at 1500 × g for 10 min. The supernatant was then stored at 6°C overnight. Before use, the free plumbagin was separated from liposomes by dialysis against isotonic phosphate buffered saline (pH 7.4) containing 30% PEG-400. The amount of unentrapped drug was measured spectrophotometrically at 269 nm.

Measurement of liposome size (blank liposomes) was made by negative stain (ammonium molybdate) electron micrograph and the phase transition temperature of the liposomal suspension (blank liposomes) was determined by differential scanning calorimetry (Perkin Elmer, DSC7).

**In vitro release characteristics of liposomes**

*In vitro* temperature dependent release rate profiles of bleomycin liposomes was determined by incubating the liposomes in saline at various temperatures as follows. The liposomes were diluted 2-fold with phosphate buffered saline (pH 7.4). Two mL of each sample was placed in a test tube and incubated for 15 min. in a water bath (Julabo PC 20B, Germany) maintained at constant temperatures (accuracy of ± 0.02°C). The released drug was separated from the liposomal suspension and assayed by the same method as in the liposomal drug content assay.

For plumbagin, liposomes were diluted 2-fold with phosphate buffered saline (pH 7.4) containing 30% PEG-400. Two mL of liposomal suspension was placed in test tube and incubated for 15 min. in a water bath maintained at constant temperature. The released drug was separated from liposomal suspension by centrifugation at 100,000 × g and the supernatant was analysed for drug content.
Stability studies

Vesicle stability with respect to drug leakage and drug degradation upon storage was studied at room temperature, 37°C and under refrigeration (4°C) for a period of one month. Liposomes containing a known quantity of drug were stored in amber colored vials under specified conditions of storage; samples were withdrawn weekly and the percentage of drug remaining in liposomes was determined.

In vivo studies in mice bearing tumour

Tumour Model

C57BL/6J mice of either sex, 6-8 weeks of age, weighing 20-25 g were procured from National Institute of Nutrition, Hyderabad, India. They were maintained under controlled temperature and humidity with sterile paddy husk as bedding and food and water ad libitum. The mouse tumour melanoma B16F1 was obtained from Department of Radiobiology, Kasturba Medical College, Manipal, and was propagated by serial transplantation in the dorsal skin of mice. For experiments, $5 \times 10^5$ viable tumour cells were injected intradermally on the dorsal skin. Once the tumour became palpable, diameters in three perpendicular planes ($D_1$, $D_2$, $D_3$) were measured on alternate days using a vernier caliper (Uma Devi and Rao 1993). The tumour volume ($V$) was calculated from the formula:

$$V = \frac{\pi \cdot (D_1 \cdot D_2 \cdot D_3)}{6}$$

Tumours measuring $100 \pm 10 \text{ mm}^3$ (8-12 days after inoculation) were taken for the experiments.

Treatment

The mice were anaesthetized by intraperitonieal injection of ketamine (50 mg/kg body weight) and diazepam (0.5 mg/mouse). For each drug formulation, mice were divided into nine groups of 10 animals each (Table 20 and 21). The animals belonging to the treatment groups were injected intravenously by tail vein with an appropriate formulation using a 27-gauge needle. The dose of free and liposomal bleomycin was 10 mg.kg$^{-1}$ while for free and liposomal plumbagin, the dose was 6 mg.kg$^{-1}$. Immediately after
drug administration, localised hyperthermia treatment was applied either for 30 min. or 1 h at 43 °C.

For hyperthermia treatment, a thermostat controlled circulating water bath with a overall dimension of 535 X 330 X 320 mm, bath opening of 360 X 300/150 mm and a bath liquid capacity of 14-20 liter (Julabo PC 20B, Germany) was used. Distilled water was used as the bath liquid. The set temperature was achieved and stabilised within 10 minutes. The anaesthetized mice were placed on a perspex tray with longitudinal slits. The tumours were pulled out through the slits in such a way that only the tumours protruded out to the water below the tray, while the animal remained above the tray (Fig. 44). Thus the tumours could be heated without affecting the other parts of the body. The tray was then placed in the water bath with the tumours immersed 1.5 to 2.0 cm below the surface. Five to seven tumours were heated at a time. A table fan was used to ventilate the air so as to control humidity above the water bath. The experimental set up for the hyperthermia treatment is shown in Fig. 45.

All the temperature measurements were done using copper constant thermocouple microprobes. The rectal temperature was measured using a rectal probe (RET-3, 1.0 cm long, Sensortek, USA). The intratumour temperatures at three points i.e. at the base, center and outer periphery of the tumour, were measured using hypodermic needle microprobes (29 gauge, 0.33 mm diameter, 1.0 cm long) in one of the animals from each group. The temperature of the water bath was also continuously monitored using a microprobe. All the microprobes were connected to a digital thermometer sensor (Sensortek ING., BAT, USA) bypassed through a multichannel thermocouple selector (Sensortek, SWT-5, USA).

After the treatment, the tumour response was assessed by parameters volume doubling time and growth delay. Volume doubling time is the time required to double the tumour volume from 100 mm³ to 200 mm³ while growth delay is the difference in time, in days, between the treated and untreated tumours to reach 500 mm³ from 100 mm³.
Fig. 44. Animals (mice) restrained on a Perspex tray, tumours protruding below.

Fig. 45. Experimental set up showing hyperthermia treatment to mice bearing tumour.
Results

In case of bleomycin, the liposomes formed were found to be mostly unilamellar and the size of the vesicles ranged from 0.2-1 µm, with mean size of approximately 0.3 µm (Fig. 46). However, large vesicles with diameter as high as 5 µm were also observed occasionally.

Fig. 46. Negative stain electron micrograph of large unilamellar liposomes (LUV) prepared by reverse phase evaporation method.

The phase transition temperature of the liposomes as determined by differential scanning calorimetry was found to be 41.2 °C. The liposomes encapsulated about 42% of the total bleomycin. When the ionic strength of the phosphate buffered saline is increased by ten times (phosphate buffered saline without dilution), the entrapment efficiency decreased to 29%. Comparison of the drug release profile at various temperatures revealed that maximum drug release (80%) occurred at 42 °C compared to less than 5% release at 37 °C (Fig. 47).
Fig. 47. *In vitro* release pattern of thermosensitive liposomes at various temperatures in phosphate buffered saline. Legends (▲) plumbagin SUV liposomes; (■) bleomycin LUV liposomes.

The liposomes were found to be stable at all temperatures of storage for the observed period of time. Storage at low temperature (4°C) was found to be particularly useful. The liposomes stored at 4°C retained more than 94% of the drug at the end of one-month (Table 20).

**Table 20.** Effect of storage conditions on stability of bleomycin and plumbagin thermosensitive liposomes.

<table>
<thead>
<tr>
<th>Time in days</th>
<th>4 °C</th>
<th>Room temp</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bleomycin liposomes (LUV)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>97.28 (3.11)</td>
<td>95.35 (2.14)</td>
<td>95.08 (2.89)</td>
</tr>
<tr>
<td>14</td>
<td>96.25 (2.89)</td>
<td>94.74 (2.96)</td>
<td>93.78 (2.77)</td>
</tr>
<tr>
<td>21</td>
<td>95.78 (3.02)</td>
<td>93.29 (2.59)</td>
<td>93.87 (3.45)</td>
</tr>
<tr>
<td>28</td>
<td>94.89 (3.09)</td>
<td>94.53 (3.18)</td>
<td>92.92 (2.97)</td>
</tr>
<tr>
<td><strong>Plumbagin liposomes (SUV)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>94.11 (4.98)</td>
<td>65.52 (4.35)</td>
<td>54.25 (5.25)</td>
</tr>
<tr>
<td>14</td>
<td>92.25 (3.98)</td>
<td>48.86 (6.20)</td>
<td>36.25 (4.89)</td>
</tr>
<tr>
<td>21</td>
<td>93.21 (4.57)</td>
<td>39.86 (6.91)</td>
<td>25.56 (6.51)</td>
</tr>
<tr>
<td>28</td>
<td>90.10 (4.19)</td>
<td>22.32 (5.21)</td>
<td>8.99 (2.97)</td>
</tr>
</tbody>
</table>

Each value is triplicate of three determinations. Values in parenthesis indicate ± SD (n=3)
The volume doubling time and growth delay observed for control and bleomycin treated groups is depicted in Table 21. Treatments with free bleomycin or bleomycin liposomes intravenously did not produce any significant increase in volume doubling time compared to control. Hyperthermia treatment (30 min.) however produced significant increase in volume doubling time and growth delay compared to control, free bleomycin or liposomal bleomycin (P < 0.01). Increasing the duration of hyperthermia to 1 hr further enhanced the tumour response with significant increase in volume doubling time (P < 0.001). Combination of free bleomycin and hyperthermia either for 30 min. or 1 h slightly enhanced the volume doubling time and growth delay compared to respective hyperthermia treatment (30 min. or 1 h) alone, however the difference was insignificant (P > 0.05). When the liposomal bleomycin was combined with hyperthermia (30 min.), the tumour response was enhanced with significant increase in volume doubling time (P < 0.01) and growth delay (P < 0.001) compared to free bleomycin with or without hyperthermia (30 min.). The maximum tumour response in terms of volume doubling time and growth delay was observed when hyperthermia treatment was applied for one hour in combination with thermosensitive bleomycin liposomes. Increase in volume doubling time and growth delay was significant compared to either free bleomycin or liposomal bleomycin with or without hyperthermia (30 min.).

Table 21. Antitumour efficacy of free and liposomal bleomycin with or without localised hyperthermia (43 °C) in mice bearing melanoma B16F1

<table>
<thead>
<tr>
<th>Group</th>
<th>Volume Doubling Time (Days)</th>
<th>Growth Delay (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.20 ± 0.34</td>
<td>-</td>
</tr>
<tr>
<td>Free bleomycin</td>
<td>3.91 ± 0.38</td>
<td>-</td>
</tr>
<tr>
<td>Liposomal bleomycin</td>
<td>3.84 ± 0.41</td>
<td>-</td>
</tr>
<tr>
<td>Hyperthermia alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>5.10 ± 0.46</td>
<td>2.92 ± 0.63</td>
</tr>
<tr>
<td>60 min.</td>
<td>6.85 ± 0.85</td>
<td>4.89 ± 0.78</td>
</tr>
<tr>
<td>Free bleomycin + hyperthermia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>6.28 ± 0.68</td>
<td>4.56 ± 0.80</td>
</tr>
<tr>
<td>60 min.</td>
<td>7.69 ± 0.98</td>
<td>6.36 ± 0.76</td>
</tr>
<tr>
<td>Liposomal bleomycin + hyperthermia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>8.29 ± 1.11*</td>
<td>7.02 ± 0.72*</td>
</tr>
<tr>
<td>60 min.</td>
<td>12.56 ± 1.48*</td>
<td>11.59 ± 1.12*</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.01) compared to free bleomycin + hyperthermia (30 min.)

b Significant difference (P < 0.001) compared to free bleomycin + hyperthermia (30 min.) & free bleomycin + hyperthermia (60 min.)
The negative stain electron micrograph of SUV for plumbagin is shown in Fig. 48. SUV's exhibited wide variation in size with mean diameter of the vesicles being approximately 90 nm.

Fig. 48. Negative stain electron micrograph of small unilamellar vesicles (SUV) prepared by thin film hydration and subsequent sonication.

The SUV's encapsulated about 19% of the total plumbagin. SUV's released plumbagin at higher temperatures, the release rate was however slower than LUV's of bleomycin. The amount of drug released at 42 and 46 °C was 42% and 51.1% respectively (Fig. 47). The SUV's were found to be stable only at low temperatures (4 °C). The liposomes stored at 4°C retained more than 90% of the drug at the end of one-month compared to less than 9% retention at 37°C over the same storage period. (Table 20).

The results obtained with plumbagin SUV's in tumour bearing mice were somewhat similar to bleomycin LUV's (Table 22). The maximum tumour response in terms of volume doubling time and growth delay was observed when hyperthermia treatment was applied for 1 h in combination with thermosensitive plumbagin liposomes. Increase in volume doubling time and growth delay was significant compared to either free plumbagin or liposomal plumbagin with or without hyperthermia (30 min.)
### Table 22. Antitumour efficacy of free and liposomal plumbagin with or without localised hyperthermia (43 °C) in mice bearing melanoma B16F1

<table>
<thead>
<tr>
<th>Group</th>
<th>Volume Doubling Time (Days)</th>
<th>Growth Delay (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.20 ± 0.34</td>
<td>-</td>
</tr>
<tr>
<td>Free plumbagin</td>
<td>3.23 ± 0.36</td>
<td>-</td>
</tr>
<tr>
<td>Liposomal plumbagin</td>
<td>3.26 ± 0.40</td>
<td>-</td>
</tr>
<tr>
<td>Hyperthermia alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>5.10 ± 0.46</td>
<td>2.92 ± 0.63</td>
</tr>
<tr>
<td>60 min.</td>
<td>6.85 ± 0.85</td>
<td>4.89 ± 0.78</td>
</tr>
<tr>
<td>Free plumbagin + hyperthermia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>6.60 ± 0.56</td>
<td>4.72 ± 0.69</td>
</tr>
<tr>
<td>60 min.</td>
<td>8.19 ± 0.84</td>
<td>6.86 ± 0.79</td>
</tr>
<tr>
<td>Liposomal plumbagin + hyperthermia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>8.31 ± 0.98*</td>
<td>7.32 ± 0.82*</td>
</tr>
<tr>
<td>60 min.</td>
<td>13.26 ± 1.21b</td>
<td>12.56 ± 1.20b</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.01) compared to free plumbagin + hyperthermia (30 min.)

\[ \text{\textsuperscript{b}} \text{Significant difference (P < 0.001) compared to free plumbagin + hyperthermia (30 min.) \& free plumbagin + hyperthermia (60 min.)} \]

### Discussion

Hyperthermia (42-44 °C) and chemotherapeutic agents in combination frequently results in increased cytotoxicity over that predicted for an additive effect (Hahn 1979). Several researchers have proved the effectiveness of this bimodality approach in tumour treatment. Our laboratory has shown the effectiveness of localised hyperthermia (43 °C) in combination with bleomycin for effective treatment of tumour, sarcoma-180 in mice (Uma Devi and Rao; 1993). Combination of alkylating agents (Nitromin) or antibiotics (Adriamycin, bleomycin, actinomycin D), or nitrosoureas (BCNU) with hyperthermia (42-43 °C) also enhanced the tumour response in animals (Marmor 1979; Hahn 1979)

The efficacy of this bimodality approach (chemotherapeutic agents and hyperthermia) could be increased dramatically if one could deliver the chemotherapeutic agents selectively to tumour cells and then apply localised hyperthermia to tumour site. The use of liposomes as a drug delivery system coupled with local hyperthermia has been suggested as an effective strategy to achieve the preferential release of the drug in a target area. The illustrative diagram of this approach is shown in Fig. 49.
Fig. 49. Schematic view of local drug release from “temperature sensitive” liposomes. (a) Mice bearing tumour on dorsal back. (b) When the liposomes are injected intravenously to mice and localised hyperthermia applied to tumour, the liposomes while passing through the heated area, release their contents at a rate dependant on temperature and the action of serum components. Released drug equilibrates throughout the extracellular space and is transported into cells as if injected in free form. (c) Release (leakage) of the drug from liposomes is because of the phase transition, where the phospholipid bilayers gets transformed from ordered state to disordered (fluid) state.

This approach of using thermoresponsive liposomes has been tested using various antitumour drugs in combination with local hyperthermia of the tumour and the results obtained were indicative of the preferential accumulation of the drug in heated tumours (Yatvin et al; 1981, Weinstein et al; 1980, Bassett et al 1986). The present study attempts at evaluating the antitumour effect of bleomycin and plumbagin by encapsulating them in temperature sensitive liposomes and using them in combination with localised hyperthermia for targeted delivery in the management of solid tumour, melanoma B16F1.
In case of bleomycin, the thermosensitive liposomes (LUV’s) were prepared with reverse phase evaporation method because of the larger aqueous fluid volume of the resulting vesicles. These types of vesicles are thought to exhibit higher encapsulation efficiency for hydrophilic drugs (such as bleomycin) compared to liposomes prepared by thin film hydration and subsequent sonication (Szoka and Papahadjopoulos 1978). The use of such liposomes can save the lipid and reduce the total lipid load thus offering a greater advantage for the therapeutic use of liposomes. The ratio of aqueous phase to organic phase was kept at 1:6 for maximal capture of the aqueous phase in the vesicles (Szoka and Papahadjopoulos 1978). The liposomes were found to entrap about 42% of the total bleomycin. The observed decrease in entrapment efficiency after increasing the ionic strength may be attributed to the decrease in volume of encapsulated aqueous space within the vesicles as reported by Szoka and Papahadjopoulos (1978). The liposomes were observed to be mostly unilamellar in nature with mean diameter of 0.3 μm with occasional larger ones.

In case of plumbagin, SUV’s were prepared because of the lipophilic nature of the drug. LUV’s as described above are suitable for encapsulation of hydrophilic drugs and not for lipophilic drugs. Multilamellar vesicles were first formed which were subsequently sonicated to obtain the vesicles (SUV’s) of size suitable for current application. The observed low entrapment of the drug (19%) may be attributed to the encapsulation of the drug in lipid bilayers. For enhanced entrapment in the vesicles, the drug was added to the organic phase.

The ratio of DPPC/DSPC was kept at 9:1 w/w because of the phase transition temperature of the resulting vesicles which was found to be 41.2°C. This temperature is easily obtainable by local hyperthermia for in vivo treatment of the tumours. Phospholipids, DPPC/DSPC in the 7:3 w/w ratio showed phase transition temperature more than 42 °C (Mabrey et al., 1981). For achieving larger chemotherapeutic effect of hyperthermia mediated liposome delivery with minimum side effects, it is favorable that hyperthermia temperature at which the drug release occurs is as low as possible. Therefore, in the present study, we chose DPPC/DSPC ratio at 9:1 w/w so as to obtain the phase transition temperature near the lower limit of hyperthermia temperature.
It has been reported that the heat specific drug release from the thermosensitive liposomes occurred explosively and completely in few seconds, indicating that the release rate does not change much if the heating time is longer than 1 min. (Iga et al. 1991). Therefore, as a convenient temperature dependent release test, we employed the method of incubating the liposomes at various hyperthermia temperatures for 15 min.

The results of the *in vitro* release studies for bleomycin liposomes at various temperatures indicated that the maximum release (80%) occurred at 42°C and less than 5% release occurred at temperatures less than 37°C. This may be because of the phase transition temperature of the phospholipid vesicles, which was found to be 41.2°C, in close correspondence to the reported values (Mabrey 1981). At higher temperatures, 42°C for example, the bilayers of the liposomes will be in the liquid crystalline fluid state (disordered fluid state) and the bleomycin entrapped in aqueous phase of the vesicles can easily migrate to the bulk aqueous phase (Fig. 49). At physiological temperature (37°C) however, the bilayers will be in a crystalline solid state (ordered gel state) which prevents the leakage of the entrapped drug to bulk aqueous phase.

Contrary to the LUV's of bleomycin, SUV's of plumbagin showed only a meager increase in drug release as the temperature was increased through the phase transition temperature. The released amount at 42 °C was only 42%. Smaller rate of drug release from the SUV as compared with LUV is probably due to the larger membrane lipid curvature of the SUV's or the binding of the drug with the phospholipid bilayers.

One of the major problems limiting the widespread use of liposomes is its stability, both physical and chemical. Liposome stability is thought to be affected by lipid composition and the type of liposome. The assessment of degree of leakage of bleomycin and plumbagin from the vesicles and drug decomposition were used as the parameters to ascertain the stability of the vesicles at various temperatures. The temperature selection was done on the basis of likely temperatures with which the vesicles would come in contact either during storage or on administration.

The result of the stability studies indicated that the LUV's of bleomycin prepared with DPPC/ DSPC were stable for the observed period of time. This was contrary to SUV's of plumbagin prepared with thin film hydration and subsequent sonication, which
coalesced over a period of time leading to marked drug leakage from the vesicles. The observed result may be attributed to the size of the vesicles as it has been reported that the smaller vesicles are more prone for fusion or aggregation than larger vesicles (Szoka and Papahadjopoulos, 1980). At lower temperatures, the liposomes exhibited better stability than at higher temperatures.

The dose selected for the evaluation of antitumour efficacy of bleomycin and plumbagin was 10 and 6 mg kg\(^{-1}\) body weight respectively (Uma Devi and Rao 1993; Raja Naresh et al 1996a,b,c). The hyperthermia conditions (43°C, 30 min or 1 h) were based on reports of various researchers as well as our own laboratory (Hahn 1979; Uma Devi and Rao 1993). Free bleomycin or plumbagin intravenously did not show any significant increase in the volume doubling time compared to control, which may be attributed to the rapid elimination of the drug from systemic circulation. Hyperthermia treatment alone could significantly enhance the volume doubling time compared to control or free bleomycin/plumbagin. The cytotoxic nature of the hyperthermia for tumour cells has been attributed to alterations in blood flow to tumour cells, followed by changes in pH, oxygen tension and transport properties (Hahn et al 1975; Marmor 1979). Increased sensitivity of the tumours has also been attributed to the heating rate. Wu et al (1985) using Chinese hamster cells demonstrated that faster the cells reach the final hyperthermia temperature, the higher is the thermal sensitivity to cell killing. In the present study, the desired temperature in the tumour was achieved within 5 min. after immersion in water bath indicating a fast heating rate. Increased duration of hyperthermia (1h) was found to be particularly useful for better tumour response. The combination of free bleomycin/plumbagin and hyperthermia did show some additive effect on antitumour activity which might be because of the interaction of hyperthermia and bleomycin/plumbagin as reported earlier, however the effect was insignificant when compared with hyperthermia alone. Liposome encapsulation of bleomycin/plumbagin in combination with hyperthermia treatment further enhanced the tumour response. The observed effect was additive as well as synergistic, which may be attributed to the selective release of the bleomycin/plumbagin to tumours from liposomes in response to hyperthermia occurring in the heated regions of the tumour and the interaction of the hyperthermia and bleomycin/plumbagin, subsequently resulting in increased cytotoxicity to tumour cells.
Although SUV's of plumbagin showed only 42% release *in vitro*, when combined with hyperthermia treatment *in vivo*, showed improved antitumour activity as evident by enhanced volume doubling time and growth delay. Presence of serum components, endocytosis or lipid-exchange with the endogenous lipids coupled with localised hyperthermia may facilitate the *in vivo* release of the drug. Moreover, the SUV's have long circulation time compared to LUV's, that gives them more opportunity to pass through the heated area repeatedly. This may lead to enhanced accumulation of drug in the heated tumour. Iga et al., (1991) has shown that the administration of cisplatin in SUV's with hyperthermia resulted in significantly higher tumour levels of drug and it was observed that the high tumour levels were maintained for a long time.

**Conclusion**

The results of the present study suggest that localised hyperthermia in combination with temperature sensitive liposome encapsulating either bleomycin or plumbagin may serve as a useful targeted drug delivery system for more effective management of melanoma B16F1. Further studies with regard to normal tissue toxicity needs to be addressed before the clinical application of this combined modality.