CHAPTER IV

Chemoprevention by liposomal DAS
4.1. Introduction

In contrast to dark skinned people whites are more prone to skin cancer and normally, one in five whites develops skin cancer during his or her life, and more than 97% of these are non-melanoma skin cancer (NMSC). Although NMSC has a low mortality, it is more common than all other cancers and has a higher incidence than lung cancer, breast cancer, prostate cancer, and colon cancer combined (American Cancer Society 2004). This "epidemic" has economic significance as well: The total cost of NMSC care in the United States is more than 600 million dollars per year (Chen et al. 2001). The rising incidence of NMSC is probably due to a combination of increased sun exposure, more frequent outdoor activities, changes in clothing style, increased longevity, and ozone depletion etc. Some studies suggest that development of NMSC, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), may indicate increased risk for internal malignancy (Spratt 1999, Karagass et al. 1999). The use of alternatives to the medicines in the form of food and food products is an effective approach for the treatment of chronic diseases, including cancer. Thus, the intervention of chemopreventive strategies for controlling genetic diseases using dietary constituents provides a valid rationale to arrest or reverse the process of carcinogenesis before invasion and metastasis occur. During the past few years, cancer chemoprevention by dietary constituents has received a great deal of attention and as a means of effective cancer control (Pezzuto 1996). Studies on the tumor inhibitory compounds of plant origin have yielded an impressive array of novel chemical agents. Besides, epidemiological studies suggest that consumption of diets constituting fruits and vegetables may reduce the risk of cancer development (El-Bayoumy et al. 1997, Reddy et al. 1997, Gescher et al. 1998). A number of dietary agents are also known to possess the anticancer properties against cancer cell lines and rodent bioassays at various sites including breast, prostate, colon and lung (Pezzuto 1996,
These include green and black tea polyphenols, I3C, sulforaphane, vitamin D, vitamin E, selenium and calcium, pomegranate etc (Kelloff et al. 1999).

In the recent past, a great deal of attention has been devoted to organosulfur compounds from garlic for their potential chemopreventive properties (Fukushima et al. 1997, Pinto & Rivlin 2001). Among these, diallyl sulfide (DAS) a sulfur containing volatile compound from garlic (Allium sativum) has received considerable emphasis. Laboratory investigations provide sufficient evidence that it reduces the incidence of a multitude of chemically induced lung, skin, colon, esophageal and forestomach neoplasia (Wargovich et al. 1992; Hu et al. 1996a, Singh & Shukla 1999, Singh & Shukla 1998, Yang et al. 2001). It has also been shown to inhibit aflatoxin B1 and NDMA induced liver preneoplastic foci in rats (Haber-Mignard et al. 1996). Several in vitro studies have also demonstrated its inhibitory effects on the tumor cells (Hageman et al. 1997, Hong et al. 2000). It has been reported that the topical application of DAS inhibit the development of tumors in both complete and two-stage model of mouse skin carcinogenesis (Singh & Shukla 1999, Singh & Shukla 1998). DAS has also shown to possess antiproliferative effects on the growth of transplantable Ehrlich ascetic tumor cells and inhibit angiogenesis in Swiss albino mice (Shukla et al. 2002). A deeper insight revealed that DAS achieves its anticancer properties by modulating phase I and II detoxifying enzymes, scavenging of free radicals and abrogating their mutagenic potential (Prasad et al. 2006, Shukla et al. 2003, Smith et al. 2000, Yang et al. 2001 and Guyonnet 1999).

Of the various options available for administration of medicaments, topical application is the most promising approach for treating skin tumors as it leads to localized effect at desired site with minimal side effects. However, retention of drugs administered by this mode is low because of extensive diffusion that is more apparent in case of small
sized molecule such as DAS. This warrants development of formulations that can modulate pharmacokinetics as well as pharmacodynamic properties of DAS thereby making it more efficacious.

Among various novel drug delivery systems, micro-particulate-based carrier systems *viz.* micro-emulsion (Spiclin *et al.* 2003), nano-emulsion (Jia-You *et al.* 2004), nanoparticles (Muller *et al.* 2002), liposomes (Betz *et al.* 2005, Kirjavainen *et al.* 1999), etc. have been reported to improve delivery of drug to the skin. Interestingly, liposome-based formulations, when employed for topical delivery, have been shown to be extremely promising for enhancement of drug penetration (Betz *et al.* 2005; Maestrelli *et al.* 2005, Cevc 1996), improved pharmacological effects (Sharma *et al.* 1994, Skalko *et al.*, 1998), decreased side effects, controlled drug release (Jia-You *et al.* 2004) and above all their own biodegradable nature (Bhatia *et al.* 2004, Drummond *et al.* 1999, Bally *et al.* 1998).

![Figure 4.1: (A) Garlic and (B) Chemical structure of diallyl sulfide (DAS)](image)
The aim of the present study was to evaluate chemo-preventive action of liposome-based topical formulations of DAS against dimethyl benz (a) anthracene (DMBA)-induced skin cancer. DMBA, a polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental pollutant that is generated during incomplete combustion of organic substances and known to have cytotoxic, mutagenic and carcinogenic effects in experimental animals as well as in humans (Guerin 1978, Dipple et al. 1984). We incorporated DAS in lipid bilayer of egg phosphatidyl-choline (PC) liposomes to achieve its slow release for prolonged duration at the site of application (Harasym et al. 1997). To the best of our knowledge no report is available till date regarding usage of liposome-based formulation of DAS against any disease including skin cancer. The efficacy of DAS against cancer was further enhanced by incorporating it in pH-sensitive liposomes, which have potential to deliver encapsulated drug to the cytosol of the cancer cells. The data of the present study also revealed that liposomal DAS was found to regulate cell cycle factors more efficiently and eventually helped in increasing the chemo-preventive properties of DAS.

4.2. Materials and Methods

4.2.1. Chemicals

All the reagents used in the study were of the highest purity available. Cholesterol was bought from Centron Research Laboratory (Mumbai, India) and used after crystallizing it three times with methanol. Egg phosphatidyl-choline (PC) was isolated and purified following the published procedure (Singleton et al. 1965). DMBA, DAS, dioleoyl phosphatidyl ethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) were purchased from Sigma Chemical Co (St. Louis, USA). Anti-p53 antibody specific for wild-type (wt) protein (clone PAb 1620, Ab-5), Anti-p53mut (clone PAb 240) and monoclonal p21/Waf1 (Ab-1) antibody were purchased from Merck India Ltd. The
horseradish peroxidase-conjugated isotypes were obtained from Bangalore Genei (Bangalore, India).

4.2.2. **Preparation of DAS bearing PC as well as pH sensitive liposomes**

Egg PC liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol), while pH-sensitive liposomes were prepared from DOPE (54 μmol) and CHEMS (36 μmol) liposomes using published method with some modifications as standardized in our lab (Owais et al. 1993). Briefly all the ingredients along with DAS (Drug:Lipid :: 1:40) were dissolved in a minimum volume of chloroform : methanol (1:1, V/V). The solvents were carefully evaporated under reduced pressure to form a thin lipid film on the wall of the round bottom flask. Finally, the traces of the organic solvents were removed by subjecting the flask to high vacuum overnight at 4 °C. Subsequently, the dried lipid film was hydrated with 2.0 ml of 150 mM sterile saline with intermittent vigorous stirring followed by sonication (1 h, 4 °C) in a bath type sonicator under N₂ atmosphere. The sonicated preparation was dialysed against normal saline for 24 hour at 4 °C in the dark, and then centrifuged at 10,000 g for 1 h at 4 °C to remove un-dispersed lipid. The liposomal preparations of DAS were used in treatment of DMBA induced skin cancer.

4.2.3. **Determination of intercalation efficiency of DAS in liposomes**

The intercalation efficiency of DAS in various formulations of liposomes was estimated by HPLC method (Khan et al. 2003). A standard curve of the drug was plotted at 271 nm by determining the area under curve corresponding to known (increasing) amount of the drug. The extent of DAS entrapped in liposome was calculated from the standard curve of the drug solution that was plotted for area under curve against corresponding amount of the DAS. The
intercalation efficiency of DAS in egg PC liposome (Lip-DAS), pH-sensitive liposome (pH-Lip-DAS) and escheriosomes (EC-Lip-DAS) formulations was found out to be 90 ± 4 percent and 96.4 ± 2.6 and 98.2 ± 3.1 respectively.

4.2.4. Animals

Female Swiss albino mice of weight 22 ± 2 g were obtained from the institute's animal facility. The animals were kept in quarantine for the period of 1 week on a 12:12-h light-dark cycle and were given a standard pellet diet and water *ad libitum*. Animals were checked daily for their mortality and morbidity prior to commencement of the study and only healthy animals were included in the experiment. The techniques used for drug administration as well as sacrifice of animals were strictly performed following mandates approved by the Animal Ethics Committee (Committee for the purpose of control and supervision of Experiments on Animals, Govt. of India).

4.2.4. Treatments

Animals in the resting phase of hair cycle were taken for the study. Hairs of the animals were removed from the interscapular region over an area of 2cm² using electric clippers that were not lubricated with any oil or grease. The skin of the shaven dorsal portion of the mice was exposed to DMBA (52μg in 200 μl acetone) that was applied topically three times a week for 12 weeks. All the formulations of DAS (250μg) were applied within 1 hour after exposure with DMBA three times a week for 12 weeks. The animals were divided into 9 groups each comprising 15 animals as follows:
## Groups and Treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>Untreated Control (Normal)</td>
</tr>
<tr>
<td>Group II</td>
<td>DMBA + Acetone</td>
</tr>
<tr>
<td>Group III</td>
<td>DMBA + Cream</td>
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<tr>
<td>Group IV</td>
<td>DMBA + Sham PC liposomes in cream (Sham-Lip)</td>
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<tr>
<td>Group V</td>
<td>DMBA + Sham pH-sensitive liposomes in cream (Sham-pH-Lip)</td>
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<tr>
<td>Group VI</td>
<td>DMBA + Free DAS (DAS)</td>
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<tr>
<td>Group VII</td>
<td>DMBA + PC-DAS liposomes in cream (Lip-DAS)</td>
</tr>
<tr>
<td>Group VIII</td>
<td>DMBA + pH-sensitive-DAS liposomes in cream (pH-Lip-DAS)</td>
</tr>
<tr>
<td>Group IX</td>
<td>Cream + (No DMBA)</td>
</tr>
</tbody>
</table>

### 4.2.5. Tumor measurements

The diameters of the tumors were measured using a Vernier Caliper and the tumor volume was determined by the formula: 
\[ V = D \times d^2 \times \pi / 6 \]
where \( V \) = tumor volume, \( D \) = biggest dimension, and \( d \) = smallest dimension.

### 4.2.5. Preparation of nuclear fraction

The skin/tumor tissues were removed from experimental mice with sharp scalpel blades. The tissue samples were placed on ice and fat was scrapped off before further processing. Finally, the samples were homogenized and the nuclear fraction was prepared according to published method (Serpi et al. 1999).

### 4.2.6. Western Blotting

The nuclear fraction was analyzed for the presence of p53wt as well as p53mut using western blotting method (Towbin et al. 1979). Briefly, protein content of the homogenate was estimated by the
routine method using BSA as a standard (Lowry et al. 1951). Proteins (30μg/well) were resolved under nondenaturing conditions on PAGE for p53wt and on 10 % SDS-PAGE gels for p53mut and electroblotted onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk and probed with appropriate antibodies [i.e., anti-p53wt (clone MAb 4H5), anti-p53mut (clone PAb 240) and monoclonal anti-p21/Waf1 (Ab-1) antibody] at dilutions recommended by the suppliers. Immunoblots were detected by horseradish peroxidase-conjugated anti-mouse IgG using chromagen 3, 3'-diaminobenzidine tetrahydrochloride. To quantify equal loading, membranes were reprobed with β-actin antibody. Data are presented as the relative pixel density of each bands normalized to band of β-actin. The intensity of the bands was quantitated using Alpha Image Analysis software on Alpha Image Gel Documentation System.

4.3. Results

In the present study we evaluated anticancer properties of various liposomal preparations of DAS. Both pH-sensitive as well as conventional egg PC liposomal formulations of DAS inhibited significant number of DMBA induced tumors incidences as compared to free form of DAS in model animals (Fig. 4.2). The liposomal DAS formulations were also successful in delaying onset of tumorogenesis. As depicted in Fig. 4.3, the onset of tumorogenesis was delayed for a period of more than one week in the mice treated with liposomal formulations (Lip-DAS or pH-Lip-DAS) formulations in comparison to animals that were treated with free form of DAS (p<0.01). Besides delaying onset of tumorogenesis, the treatment with both pH-sensitive as well as egg PC liposomes ensued in significant reduction in total numbers of DMBA induced papillomas. As shown in Fig. 4.4(A), the mean numbers of tumors per mouse were significantly reduced in the group of animals treated with Lip-DAS, (mean value 5.84, p<0.001)
and pH-Lip-DAS, (mean value 3.91, $p<0.001$) in comparison with the animals treated with free form of DAS (mean value 10.6).

We also measured regression in the tumor volume after treatment with various forms of DAS. The mean tumor volume per mouse was significantly lower in pH-Lip-DAS treated animals (62.42 mm$^3$ $p<0.001$) while it was 168.60 mm$^3$ ($p<0.001$) in Lip-DAS treated animals. The treatment with free form of DAS reduced mean tumor volume to 309.61 mm$^3$ ($p<0.001$). The mean tumor volumes recorded in the control animals (Gps II and III) were 1048.98 mm$^3$ and 1041.37 mm$^3$ respectively (Fig. 4.4B). The animals treated with Sham-Lip and Sham-pH-Lip (Gps. IV and V) had tumor dimensions of 984.99 and 945.88 mm$^3$ respectively.

Tumor growth inhibition was used as another parameter to assess efficacy of liposomised DAS against DMBA induced papilloma formation. The tumor growth inhibition, in comparison to the DMBA exposed untreated group, was 94 % ($p<0.001$) in the group of animals treated with pH-Lip-DAS, while it was 84 % in Lip-DAS treated animals. Free form of DAS could cause 70 % inhibition in tumor growth (Fig. 4.4C).

The Kaplan-Meier curve (Fig. 4.5) shows the augmentation of anticarcinogenic effects of liposomised DAS against DMBA induced tumorogenesis in terms of survival of tumor free animals at different time intervals. Treatment with acetone, cream base, Sham-Lip, Sham-pH-Lip and free DAS could not prevent tumor incidence in DMBA exposed animals. The liposomised DAS showed tremendous increase in chemo-preventive efficacy over its free form against DMBA induced tumorogenesis. The treatment caused -23 % and 34 % of animals completely free of tumor incidences upon treatment with Lip-DAS and pH-Lip-DAS respectively.
4.3.1. Western Blot analysis

Exposure to carcinogen DMBA induced down-regulation of p53wt protein in the treated animals (Gp. II and Gp. III) in comparison with normal healthy mice (Gp. I, Fig. 5a, lanes 1, 2 and 3). Treatment with Sham-Lip and Sham-pH-Lip (Gp. IV and V) reduced DMBA induced downregulation of p53wt (although not significantly) in comparison to animals that were treated with acetone and cream (Gp. II and III). As depicted in Fig. 4.6(A), a comparatively high expression of p53wt protein was recorded in the animals treated with liposomal as well as free formulations of DAS (147 % increase in expression in the group of animals treated with pH-Lip-DAS, 84 % in the animals treated with Lip-DAS liposomes and 23 % in the animals treated with free form of DAS) over the group of animals treated with acetone and cream. This directly suggests that liposomal formulation of DAS plays a determining role in regulation of the expression of p53wt protein and inhibition of DMBA-induced neoplastic changes. The neat cream, used as a base in the preparation of various formulations, could not normalize p53wt expression in DMBA exposed mice. This observation was same in animals not given any kind of chemo-preventive treatment and hence rules out any ameliorating role of the cream base. Exposure of animals to DMBA ensued in upregulation of p53mut protein (Fig. 4.6B, lanes 2 and 3). Treatment with various forms of DAS aborted DMBA induced upregulation of p53mut. As depicted in the Fig. 4.6(B), the level of p53mut was reduced to normal level in the animals treated with pH-Lip-DAS. The treatment with Lip-DAS ensued in 64 % reduction of p53mut level, while there was 40 % reduction in the animals treated with free form of DAS (Gp. IV). Surprisingly, treatment with Sham-Lip and Sham-pH-Lip (without DAS) also nullify DMBA induced over-expression of p53mut although not significantly. These results clearly show DAS mediated down-regulation of p53mut, which was more apparent upon its intercalation in various forms of the liposomes used in the present study.
We also studied the effect of liposomised DAS on the expression of p21/Waf1, which got transcriptionally up-regulated in the presence of p53wt. Immunoblot analysis showed increased expression of p21/Waf1 in liposomal DAS treated groups. As shown in Fig. 4.7, there was 93 % increase in the expression of p21/waf1 in the group of animals treated with pH-Lip-DAS, while 66 % increment recorded in the animals treated with Lip-DAS liposomes (Gp. V), and 46 % increase occurred in the group of animals treated with free form of DAS when compared with the DMBA exposed animals that were treated with acetone and cream.
Figure 4.2: Swiss albino mouse (Shaved dorsal skin)
Figure 4.3.1: Skin with tumors in mice exposed to DMBA followed by Acetone.

Figure 4.3.2: Skin with tumors in mice exposed to DMBA followed by Cream.

Figure 4.3.3: Skin with tumors in mice exposed to DMBA followed by Shan-Lip.
Figure 4.3.4: Skin with tumors in mice exposed to DMBA followed by Sham-pH-Lip

Figure 4.3.5: Skin with tumors in mice exposed to DMBA followed by Free DAS.

Figure 4.3.6: Skin with tumors in mice exposed to DMBA followed by Lip-DAS.
Figure 4.3.7: Skin with tumors in mice exposed to DMBA followed by pH-Lip-DAS.
Figure 4.4: Chemo-preventive effect of various formulations of DAS on onset of mouse skin tumorogenesis.

I, Untreated Control (Normal); II, DMBA + Acetone; III, DMBA + Cream; IV, DMBA + Sham-Lip; V, DMBA + Sham-pH-Lip; VI, DMBA + Free DAS; VII, DMBA + Lip-DAS; VIII, DMBA + pH-Lip-DAS; IX, Cream base only. a\textit{p}<0.01 (free DAS), b\textit{p}<0.001 (Lip-DAS and pH-Lip-DAS) versus vehicle control groups (II-V), c\textit{p}<0.001 (pH-Lip-DAS and Lip-DAS) versus free DAS (VI).
Figure. 4.5(A): **Effect of liposomised DAS mediated chemoprevention on the development of average number of tumors per mouse.**

I, Untreated Control (Normal); II, DMBA + Acetone; III, DMBA + Cream; IV, DMBA + Sham-Lip; V, DMBA + Sham-pH-Lip; VI, DMBA + Free DAS; VII, DMBA + Lip-DAS; VIII, DMBA + pH-Lip-DAS; IX, Cream base only. *p<0.001 (free DAS, Lip-DAS, pH-Lip-DAS) versus vehicle control groups (II-V), †p<0.001 (Lip-DAS and pH-Lip-DAS) versus free DAS, ‡p< 0.01 (pH-Lip-DAS) versus Lip-DAS.
Figure 4.5(B): *Chemo-preventive effects of various formulations of DAS on average tumor size.*

The chemo-preventive efficacy of various forms of DAS was assessed by measuring size of the tumors using a caliper. I, Untreated Control (Normal); II, DMBA + Acetone; III, DMBA + Cream; IV, DMBA + Sham-Lip; V, DMBA + Sham-pH-Lip; VI, DMBA + Free DAS; VII, DMBA + Lip-DAS; VIII, DMBA + pH-Lip-DAS; IX, Cream base only. *p<0.001 versus vehicle controls (II-V), b p<0.001 (Lip-DAS and pH-Lip-DAS) versus free DAS. c p<0.001 (pH-Lip-DAS) versus Lip-DAS.
Figure 4.5(C): Percent-inhibition of tumor growth by various formulations of DAS.

The tumor growth inhibition was calculated by comparing the average size of the tumor induced in animals that were treated with vehicle controls. \(^{a}p<0.001\) versus (Free DAS). \(^{b}p<0.001\) versus Lip-DAS.
Figure 4.6: Effect of various formulations of DAS on survival of tumor-free animals.

Kaplan-Meier curve showing effect of various formulations of DAS in terms of percentage of tumor free animals at different time intervals. The analysis was made on weekly basis.
Figure 4.7(A): Effect of various formulations of DAS on the expression of p53wt in mouse skin tumors.

Lane 1, untreated; lane 2, DMBA + Acetone; lane 3, DMBA + Cream; lane 4, DMBA + Sham-Lip; lane 5, DMBA + Sham-pH-Lip; lane 6, DMBA + Free DAS; lane 7, DMBA + Lip-DAS; lane 8, DMBA + pH-Lip-DAS and lane 9, Cream base only with no DMBA.
Figure. 4.7(B): Effect of various formulations of DAS on the expression of p53mut in mouse skin tumors.

Lane 1, untreated; lane 2, DMBA + Acetone; lane 3, DMBA + Cream; lane 4, DMBA + Sham-Lip; lane 5, DMBA + Sham-pH-Lip; lane 6, DMBA + Free DAS; lane 7, DMBA + Lip-DAS; lane 8, DMBA + pH-Lip-DAS and lane 9, Cream base only with no DMBA.
Figure 4.8: Effect of various formulations of DAS on the expression of p21/Waf1 in mouse skin tumors.

Lane 1, untreated; lane 2, DMBA + Acetone; lane 3, DMBA + Cream; lane 4, DMBA + Sham-Lip; lane 5, DMBA + Sham-pH-Lip; lane 6, DMBA + Free DAS; lane 7, DMBA + Lip-DAS; lane 8, DMBA + pH-Lip-DAS and lane 9, Cream base only with no DMBA.
4.4. Discussions

A considerable emphasis is being laid upon the use of dietary constituent DAS, an organosulphur compound of garlic, to prevent and cure cancer in rodent tumor models (Arora et al. 2006, Prasad et al. 2006, Arora et al. 2004, Yang et al. 2001, Pinto et al. 2001, Singh et al. 1999, Singh et al. 1998, Haber et al. 1996, Wargovich et al. 1988, Sparnins et al. 1988). Although effective, the chemo-preventive properties of DAS are far from ideal to make it a potential future chemo-preventive agent against skin cancer. The small size of the molecule probably results in poor accumulation and did not allow attainment of effective therapeutic concentration at the tumor site. Earlier studies showed that localized drug delivery to the site of skin tumor could be significantly enhanced through the use of liposome-based drug carriers (Betz et al. 2005; Maestrelli et al. 2005, Cevc 1996). The present study focuses on evaluation of chemo-preventive efficacy of DAS upon its incorporation in egg PC as well as pH-sensitive liposomes and their potential against skin cancer in model animals.

The data of the present study establish higher efficacy of both Lip-DAS (conventional egg PC) and pH-Lip-DAS (pH-sensitive) liposomes, which was assessed on the basis of their ability to delay onset of tumor induction, reduction in total numbers of tumor papilloma formation and survival of the treated animals etc. Lip-DAS liposomes showed an efficient (84%) suppression of tumor growth, while its pH-sensitive liposomal formulation was found to be more effective and induced around 94 % tumor suppression (as comparison to the untreated control group). This clearly suggests that liposomisation offers a new and effective option to increase the chemo-preventive and anticarcinogenic potential of DAS in cancer therapy. The increased efficacy of liposomised DAS could be attributed to the fact that liposomes act as sustained release system allowing greater accumulation of drug molecules at the tumor site than that achieved

The liposomised diallyl sulfide exerts its chemo-preventive action by modulating apoptotic factors present in the cytosol of the cancer cells therefore its access to the cytosol is crucial for anticancer activity. The pH-sensitive liposomes were found to be more effective as compared to egg PC liposome as former release their contents into cytoplasm of the target cells following their degradation in endo-lysosomal compartment. In fact, pH sensitive liposomes undergo phosphatidyl-ethanolamine (main constituent of these liposomes) mediated phase transition at acidic pH, thereby delivering their content to the cytosol of the tumor cells. The polar head group of PE gets less hydrated as compared to repulsive hydration layer associated with the head group of PC. Thus PE provides a more hydrophobic bilayer surface that is susceptible to energetically more favorable interbilayer interactions. The phospholipid not only facilitate the close approximation of bilayers, it may also be directly involved in the merging process. In this context PE can form the hexagonal H_{11} phase, the formation of which involves the development of non-lamellar structure an intermediate in membrane fusion. The operative mechanism seems to form the basis of the observed higher efficacy of pH sensitive liposomes over egg PC neutral liposomes (Sergio et al. 2001, Yatvin et al. 1980). Moreover the higher effectiveness of pH-sensitive liposome can also be justified on the premise that sites of greatest acidity in tumors are often most distant from the tumor microvasculature, incidentally conventional liposomes often fail to reach such locations (Huang et al. 1992, Dellian et al 1996 and Helmlinger et al. 1997), while pH-sensitive liposomes overcome this problem.

The usage of liposomes as carrier of anticancer agents including DAS has added advantage as fatty acyl chains of phospholipids may also impart anticancer effect against various cancers. For example,
liposomes composed of phosphatidylcholine (PC) with 18:0 in the sn-1 position and one of the following fatty acids in the sn-2 position: 18:0, 18:1 omega 9 (oleic), 18:3 omega 3 (alpha-linolenic), 20:4 omega 6 (arachidonic), 22:6 omega 3 (docosahexaenoic) have been shown to possess antitumor effects in vivo, leading to enhanced longevity of the tumor-bearing host (Jenski et al. 1995). It has been reported that polyunsaturated phosphatidylcholine (PC) and phosphatidylserine (PS) induce growth inhibition, differentiation and apoptosis in Caco-2 cells (Hossain et al. 2006). Our preliminary studies show that egg PC used in the present study is having 18:0, 18:1 and 16:0, 16:1 fatty acid analogs (data not shown). Similarly, pH-sensitive liposomes also contain 18:1 oleic acids. Besides intrinsic anticancer effect of DAS, presence of unsaturated fatty acids in liposomes could also be considered as potential component to enhance anticancer property of DAS liposome formulation. The data of the present study shows some level of anticancer activity by sham liposome preparation (with no DAS). However the effect was not substantial and could be attributed to the fact that amount of the anticancer fatty acids present in various phospholipids used for preparation of liposomes never touched optimum threshold levels required for their anticancer effect, had we used phospholipids that were exclusively consisted of fatty acyl chains with potent anticancer activity. Nevertheless, the present study clearly advocates the notion that in future studies we can always use tailor-made phospholipids which are equipped with desired fatty acyl chains having intrinsic anticancer properties. This will certainly make liposome-based formulations potential candidate for drug delivery system against cancer including skin papilloma.
Figure 4.9: Potential fate of pH-sensitive liposome upon interaction with target cell.

Upon approaching to a target cell, liposomes can remain bound at the cell surface, accumulate in coated or non-coated invaginations or internalized by receptor mediated uptake. In acidic environment of endo-lysosomal compartment, pH sensitive phospholipids of liposome undergo phase transition (bilayer to \( \text{H}_\text{II} \) form) and fuse with endo-lysosome membrane thereby releasing their contents directly into the cytoplasm of the target cell. (a), liposomes can be delivered to lysosomes (c) where they and their contents may be degraded by lysosomal peptidases and hydrolases. Following acidification of the endosomal lumen, pH-sensitive liposomes are designed to either fuse with the endosomal membrane (e), releasing their contents directly into the cytoplasm, or become destabilized and subsequently destabilize the endosomal membrane (d) resulting in leakage of the endosomal contents into the cytosol. Receptors may be recycled back to the cell surface (b) or targeted for degradation in the lysosome (c). Enhance anticancer property of DAS liposome formulation. The data of the present study shows some level of anticancer activity by sham liposome preparation (with no DAS). However the effect was not substantial and could be attributed to the fact that amount of the anticancer fatty acids present in various phospholipids used for preparation of liposomes never touched optimum threshold levels required for their anticancer effect, had we used phospholipids that were exclusively consisted of fatty acyl chains with potent anticancer activity. Nevertheless, the present study clearly advocates the notion that in future studies we can always use tailor-made phospholipids which are equipped with desired fatty acyl chains having intrinsic anticancer properties. This will certainly make liposome-based formulations potential candidate for drug delivery system against cancer including skin papilloma.
Chapter 4

Chemoprevention by liposomal DAS

In the next phase of study, we deliberated effect of liposomised DAS on various cell cycle regulating factors. The tumor suppressor gene p53 is regarded as a key factor in maintaining the balance between cell growth and cell death (Agarwal et al. 1998, Mowat et al. 1998). We have earlier reported DAS mediated modulation of p53 in DMBA-induced skin tumors in Swiss albino mice (Arora et al. 2004). The importance of p53 gene can be drawn from the fact that this gene is mutated in ~80% of all human malignancies (Hollstein et al. 1991). Because of its role in regulation of cell cycle, alterations in p53 levels are critical in carcinogenesis. High levels of activated and stabilized p53 protein accumulate in the nucleus in response to various forms of cell stresses, including DNA damage (Vousden et al. 2002, Levine et al. 1991). Several nuclear localization signals in the C-terminus of p53 facilitate its transport to the nucleus where it is required in regulation of transcription. Having entered the nucleus, regulatory mechanisms exist to control the export of p53 back out to the cytoplasm, which is required for the degradation of p53 (Stommel et al. 1999). Activated p53 can induce cell cycle arrest, DNA repair processes, and apoptosis. These cellular outcomes are thought to minimize the accumulation of deleterious mutations that could eventually contribute to a given malignant phenotype (Lane 1992). Keeping above facts into consideration the role of p53 in the cytoplasm is clearly secondary to its nuclear function, and nuclear localization is essential for p53 activity. Indeed failure of wild type p53 to localize to the nucleus, either due to defects in the ability to enter the nucleus or hyperactive nuclear export, appears to contribute to the inactivation of p53 in a number of tumors (Stommel et al. 1999, Sengupta et al. 2000 and Lu et al. 2000). In tumors, loss of p53wt prevents the activation of this growth control pathway (Burns et al. 1991). The failure to induce transcriptionally active p53wt plays a role in the unregulated growth of the tumors (el-Deiry 1994). Because the balance between p53wt and p53mut determines the fate of the cell, many chemo-preventive agents are known to exert their anticancer effects by modulating
expression level of these molecules (Schwartz et al. 1999 and Schwartz et al. 1993). The results of the present study showed liposomal formulations of DAS in increased up-regulation of p53wt and down-regulation of p53mut. In fact, it is the intrinsic property of DAS to modulate the balance between wild and mutated p53 protein expression. The effect of DAS on p53wt and p53mut gets more apparent upon its liposomisation in neutral as well as pH-sensitive liposomes.

The up-regulation of p53wt by chemo-preventive agents is also responsible for the transcriptional induction of p21/Waf1 by directly interacting with its regulatory elements (el-Deiry et al. 1994). As observed in the present study, liposomisation of DAS in egg-PC or pH-sensitive liposomes ensued in up-regulation of p21/Waf1 as compared to free form of the drug. The effect is more prominent in pH-sensitive liposomes as compared to neutral egg PC liposomes.

As the composition of the liposome is similar to the components of cell membranes, they are likely to get absorbed by skin (Gregoriadis 1994). To further increase the effect, we formulated cream-based liposomal formulations of DAS, which ought to impart better retention and penetration of the compound. When a liposome-bearing gel or cream is applied to the skin, the deposited liposomes begin to merge with the cellular membranes. In the process, the liposomes release their payload of active materials into the cells. As a consequence, not only does this ensue in specific drug delivery of the active form of the drug directly into the target cells, the delivery also takes place over a longer period of time (Padamwar et al. 2006).

The observed better efficacy of liposomised DAS can be attributed to the liposome-mediated enhanced penetration, retention and accumulation of the drug at the tumor site. Finally we conclude that liposomal formulations of DAS can be a promising strategy for cancer treatment. Liposomal formulations not only overcome higher
elimination of the drug from tumors, rather ability of liposome to accommodate several thousand molecules in single vesicle entity that in turn help in maintaining the effective drug concentration at the tumor site.