

Potential of Diallyl Sulfide Bearing pH-Sensitive Liposomes in Chemoprevention Against DMBA-Induced Skin Papilloma

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Diallyl sulfide (DAS), an active component of garlic, possesses strong anti-neoplastic properties against various forms of cancer. In the present study, we have evaluated chemo-preventive effects of liposomized DAS (conventional egg PC and pH-sensitive liposomes) against DMBA-induced skin papilloma. Various liposome-based novel formulations of DAS (250 µg/mouse) were applied topically, after one hour of exposure to DMBA (52 µg/mouse/dose), to the animals. The animals were treated thrice weekly for the total period of 12 weeks. The efficacy of the various liposomal formulations of DAS was evaluated on the basis of parameters such as incidence of tumorigenesis and total numbers and sizes of induced tumor nodules. The liposomized DAS formulations also were assessed for their effect on the expression of p53wt, p53mut, and p21/Waf1. The results of the present study showed that liposomized DAS could effectively delay the onset of tumorigenesis and reduce the cumulative numbers and sizes of tumor papillomas in treated mice. Treatment of DMBA-exposed animals with the liposomal formulation of DAS ensued in up-regulation of p53wt and p21/Waf1, while levels of p53mut expression reduced down. The promising chemo-preventive nature of liposomal DAS may form the basis for establishing effective means of controlling various forms of cancer, including skin papilloma.

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INTRODUCTION

Garlic (*Allium sativum*) has been shown to possess potential health benefits (lipid lowering, antimicrobial, chemo-preventive, and anticarcinogenic properties, for example) since the beginning of recorded history and is probably one of the most widely studied medicinal plants (1,2). The chemotherapeutic and antitumor activity associated with garlic has been attributed to the presence of various organosulfide-based active compounds including DAS (Figure 1), (3,4,5,6,7,8). Our lab, as well as other investigators, has demonstrated the anticarcinogenic properties of DAS against chemically induced carcinogenesis in model animals (3,9,10). DAS has been

found to be effective against various forms of cancer affecting skin, lung, esophagus, colon, liver, and others (3,9,10,11). A deeper insight revealed that DAS achieves its anticancer properties by modulating phase I and phase II detoxifying enzymes, scavenging of free radicals, and abrogating their mutagenic potential (4,12,13,14,15).

Of the various options available for the administration of medications, topical application is the most promising approach for treating skin tumors as it leads to a localized effect at the 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 molecules 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, microparticulate-based carrier systems such as micro-emulsion (16), nano-emulsion (17), nanoparticles (18), liposomes (19,20), and others have been reported to improve the delivery of drugs to the skin. Interestingly, liposome-based formulations, when employed for topical delivery, have been shown to be extremely promising for enhancing drug penetration (19,21,22), improving pharmacological effects (23,24), decreasing side effects, controlling drug release (17), and, above all, their own biodegradable nature (25,26).

The aim of the present study was to evaluate chemo-preventive action of liposome-based topical formulations of DAS against dimethyl benz (a) anthra-

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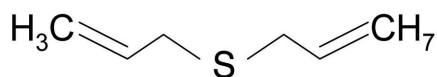


Figure 1. Chemical structure of DAS

cene (DMBA)-induced skin cancer. DMBA, a polycyclic aromatic hydrocarbon (PAH), is an ubiquitous environmental pollutant generated during incomplete combustion of organic substances and known to have cytotoxic, mutagenic, and carcinogenic effects in experimental animals as well as in humans (27,28). We incorporated DAS in a lipid bilayer of egg phosphatidyl-choline (PC) liposomes to achieve its slow release for prolonged duration at the site of application (29). To the best of our knowledge, no report is available to 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 the potential to deliver the encapsulated drug to the cytosol of the cancer cells. The data of the present study also reveal that liposomal DAS was found to regulate cell cycle factors more efficiently and eventually helped in increasing the chemopreventive properties of DAS.

MATERIALS AND METHODS

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 (30). DMBA, DAS, dioleoyl phosphatidyl ethanolamine (DOPE), and cholesteryl hemisuccinate (CHEMS) were purchased from Sigma Chemical Company (St. Louis, MO, 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 Limited (Mumbai, India). The horseradish peroxidase-conjugated isotypes were obtained from Bangalore Genei (Bangalore, India).

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 one 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 the killing of the animals were strictly performed following mandates approved by the Animal Ethics Committee (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

Preparation of DAS-Bearing 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) using published method with some modifications as standardized in our lab (31). 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 evaporated carefully 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 (one hour, 4°C) in a bath type sonicator under N_2 atmosphere. The sonicated preparation was dialyzed

against normal saline for 24 h at 4°C in the dark, and then centrifuged at 10,000g for one hour at 4°C to remove undispersed lipid. The liposomal preparations of DAS were used in treatment of DMBA-induced skin cancer.

Determination of Intercalation Efficiency of DAS in Liposomes

The intercalation efficiency of DAS in various formulations of liposomes was estimated by HPLC method (32). A standard curve of the drug was plotted at 271 nm by determining the area under curve corresponding to the 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 the area under curve against the corresponding amount of the DAS. The intercalation efficiency of DAS in egg PC liposome (Lip-DAS) and pH-sensitive liposome (pH-Lip-DAS) formulations was found out to be 90 ± 4 percent and 96.4 ± 2.6 percent respectively.

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 2 cm^2 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 (52 μg in 200 mL acetone) that was applied topically three times a week for 12 weeks. All the formulations of DAS (250 μg) were applied within one hour after exposure with DMBA three times a week for 12 weeks. The animals were divided into nine groups each comprising 15 animals as follows: Group I, Untreated Control (Normal); Group II, DMBA + Acetone; Group III, DMBA + Cream; Group IV, DMBA + Sham PC liposomes in cream (Sham-Lip); Group V, DMBA + Sham pH-sensitive liposomes in cream (Sham-pH-Lip); Group VI, DMBA + Free DAS (DAS); Group VII, DMBA + PC-DAS liposomes in cream (Lip-DAS); Group

VIII, DMBA + pH-sensitive-DAS liposomes in cream (pH-Lip-DAS); and Group IX, Cream + (No DMBA).

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.

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 scraped off before further processing. Finally, the samples were homogenized and the nuclear fraction was prepared according to published method (33).

Western Blotting

The nuclear fraction was analyzed for the presence of p53wt as well as p53mut using the Western blotting method (34). Briefly, the protein content of the homogenate was estimated by the routine method using BSA as a standard (35). Proteins (30 $\mu\text{g}/\text{well}$) were resolved under nonreducing conditions on PAGE for p53wt and on ten percent SDS-PAGE gels for p53mut and electroblotted onto nitrocellulose membranes. The blots were blocked overnight with five percent 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 band normalized to a band of β -actin. The intensity of the bands was quantitated

using Image Analysis software on an Image Gel Documentation System.

Statistical Analysis

One-way ANOVA was used for comparing the mean values of tumor numbers and tumor volume between different treated groups after ascertaining the homogeneity of variance between treatments. Post hoc analysis for comparing the two groups was done using the Least Statistical Difference (LSD) technique. The Kaplan-Meier method was used to estimate tumor free survival and differences were analyzed by the log-rank test.

RESULTS

In the present study, we evaluated anticancer properties of various liposomal preparations of DAS. Both pH-sensitive and conventional egg PC liposomal formulations of DAS inhibited a significant number of DMBA-induced tumor incidences as compared with the free form of DAS in model animals. The liposomal

DAS formulations also were successful in delaying onset of tumorigenesis. As depicted in Figure 2, the onset of tumorigenesis was delayed for a period of more than one week in the mice treated with liposomal formulations (Lip-DAS or pH-Lip-DAS) in comparison to animals that were treated with free form of DAS ($P < 0.01$). Besides delaying the onset of tumorigenesis, treatment with both pH-sensitive as well as egg PC liposomes resulted in significant reduction in total numbers of DMBA-induced papillomas. As shown in Figure 3A, 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 the 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

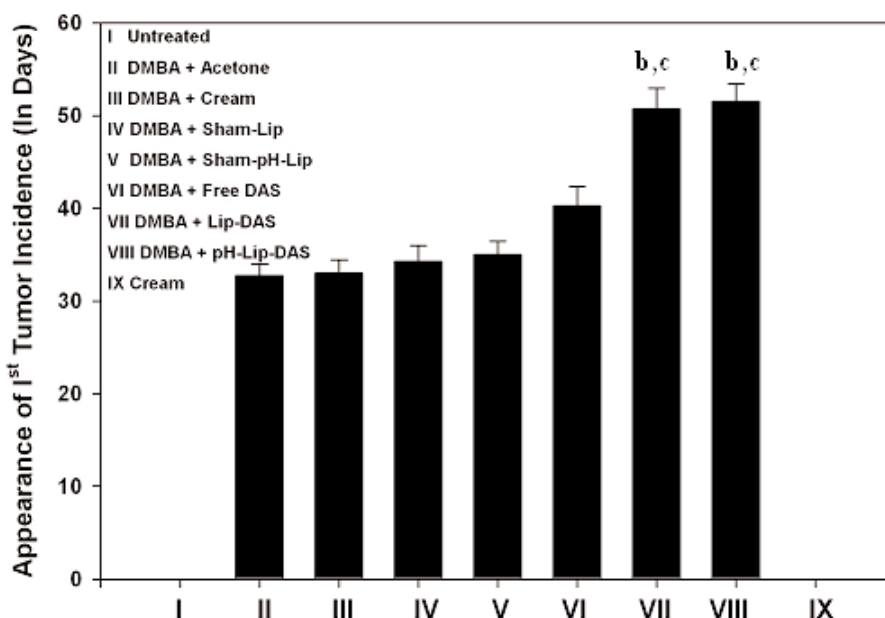


Figure 2. Chemo-preventive effect of various formulations of DAS on onset of mouse skin tumorigenesis. The animals were exposed to DMBA and subsequently treated with various forms of DAS as described in Materials and Methods. The onset of tumorigenesis was recorded as the first appearance of a DMBA-induced tumor in each group of animals. ^a $P < 0.01$ (free DAS), ^b $P < 0.001$ (Lip-DAS and pH-Lip-DAS) versus vehicle control groups (groups II-V), ^c $P < 0.001$ (pH-Lip-DAS and Lip-DAS) versus free DAS (group VI).

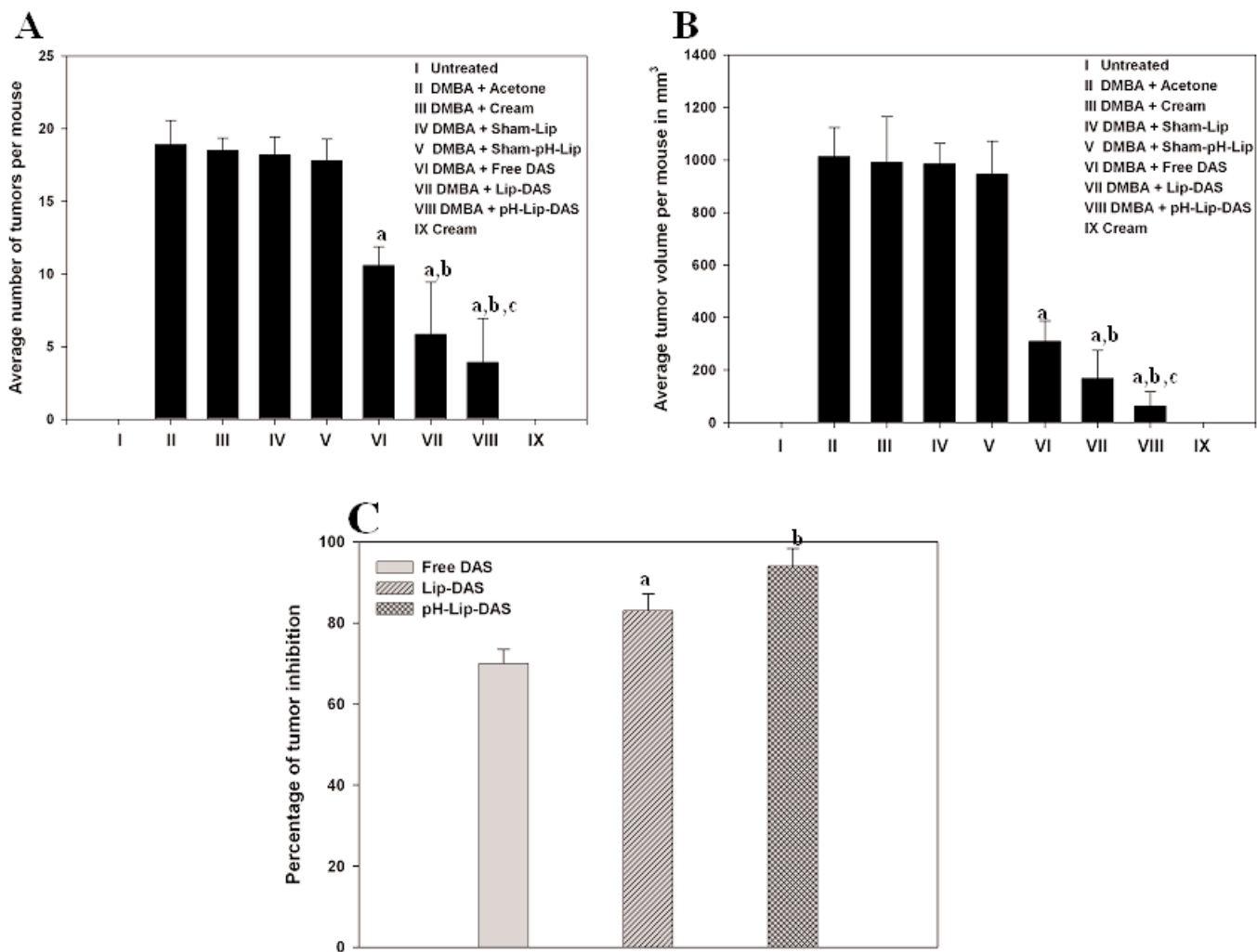


Figure 3. A. Effect of liposomized DAS mediated chemo-prevention on the development of average number of tumors per mouse. The animals were treated with various forms of DAS after their exposure to carcinogenic agent DMBA. Efficacy of various DAS liposomal formulations was assessed on the basis of their ability to prevent development of tumors. ^a*P* < 0.001 (free DAS, Lip-DAS, pH-Lip-DAS) versus vehicle control groups (groups II-V), ^b*P* < 0.001 (Lip-DAS and pH-Lip-DAS) versus free DAS, ^c*P* < 0.01 (pH-Lip-DAS) versus Lip-DAS. 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 the size of the tumors using a caliper. The tumor volume was determined by the formula as described in Materials and Methods. ^a*P* < 0.001 versus vehicle controls (groups 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 (group VII). C. Percent-inhibition of tumor growth by various formulations of DAS. The animals were treated with various forms of DAS after exposure to DMBA as described in Materials and Methods. The tumor growth inhibition was calculated by comparing the average size of the tumor induced in animals that were treated with vehicle control (acetone treatment). ^a*P* < 0.001 versus (Free DAS). ^b*P* < 0.001 versus Lip-DAS.

in pH-Lip-DAS treated animals (62.42 mm³, *P* < 0.001) while it was 168.60 mm³ (*P* < 0.001) in Lip-DAS treated animals. Treatment with the free form of DAS reduced the mean tumor volume to 309.61 mm³ (*P* < 0.001). The mean tumor volumes recorded in the control animals (groups II and III) were 1048.98 mm³ and

1041.37 mm³ respectively (Figure 3B). The animals treated with Sham-Lip and Sham-pH-Lip (groups IV and V) had tumor dimensions of 984.99 and 945.88 mm³ respectively.

Tumor growth inhibition was used as another parameter to assess efficacy of liposomized DAS against DMBA-

induced papilloma formation. The tumor growth inhibition, in comparison to the DMBA-exposed untreated group, was 94 percent (*P* < 0.001) in the group of animals treated with pH-Lip-DAS, while it was 84 percent in Lip-DAS treated animals. The free form of DAS resulted in

70 percent inhibition in tumor growth (Figure 3C).

The Kaplan-Meier curve (Figure 4) shows the augmentation of anticarcinogenic effects of liposomized DAS against DMBA-induced tumorigenesis 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 liposomized DAS showed tremendous increase in chemo-preventive efficacy over its free form against DMBA induced tumorigenesis. The treatment resulted in ~23 percent and 34 percent of animals completely free of tumor incidences upon treatment with Lip-DAS and pH-Lip-DAS respectively.

Western Blot Analysis

Exposure to carcinogen DMBA induced down-regulation of p53wt protein in the treated animals (groups II and III) in comparison with normal healthy mice (group I, Figure 5A, lanes 1, 2, and 3). Treatment with Sham-Lip and Sham-pH-Lip (group IV and V) reduced DMBA induced downregulation of p53wt (although not significantly) in comparison to animals treated with acetone and cream (groups II and III). As depicted in Figure 5A, a comparatively high expression of p53wt protein was recorded in the animals treated with liposomal as well as free formulations of DAS (147 percent increase in expression in the group of animals treated with pH-Lip-DAS, 84 percent in the animals treated with Lip-DAS liposomes, and 23 percent in the animals treated with the 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 the regulation of the expression of p53wt protein and the 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 the same as in animals not given any kind of

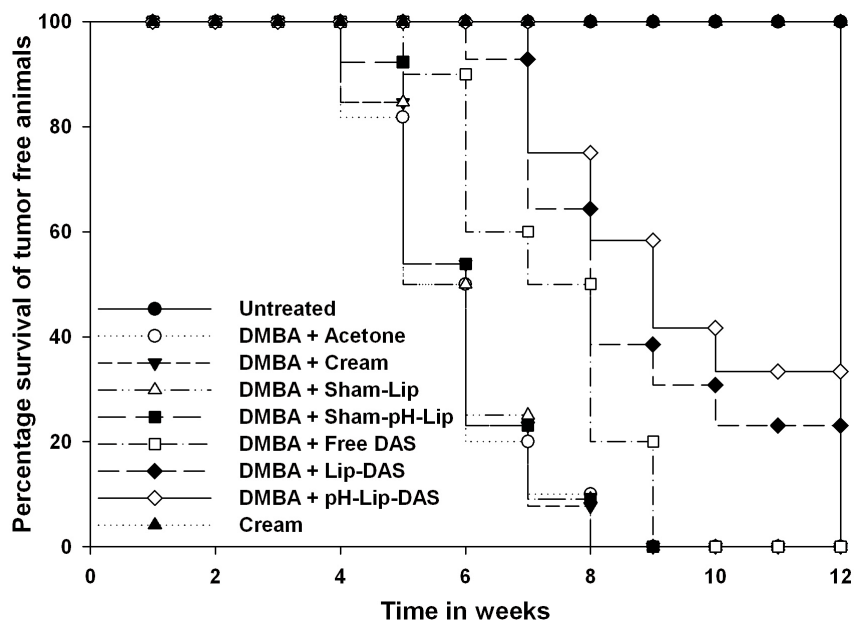


Figure 4. Effect of various formulations of DAS on survival of tumor-free animals. Kaplan-Meier curve showing effect of various formulations of DAS in terms of percentage of animals free from tumor burden at different time intervals. The study was scheduled for a period of 12 weeks as described in Materials and Methods. The analysis was made on weekly basis.

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 (Figure 5B, lanes 2 and 3). Treatment with various forms of DAS aborted DMBA induced upregulation of p53mut. As depicted in the Figure 5B, the level of p53mut was reduced to normal levels in the animals treated with pH-Lip-DAS. The treatment with Lip-DAS yielded a 64 percent reduction of p53mut level, while there was a 40 percent reduction in the animals treated with the free form of DAS (group 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 downregulation 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 liposomized DAS on the expression of p21/Waf1, which was transcriptionally upregulated in the presence of p53wt. Immunoblot analysis showed increased expression of p21/Waf1 in liposomal DAS-treated groups. As shown in Figure 6, there was a 93 percent increase in the expression of p21/waf1 in the group of animals treated with pH-Lip-DAS, while a 66 percent increment was recorded in the animals treated with Lip-DAS liposomes (group V), and a 46 percent increase occurred in the group of animals treated with the free form of DAS when compared with the DMBA-exposed animals treated with acetone and cream.

DISCUSSION

A considerable emphasis is being placed upon the use of dietary constituent DAS, an organosulphur compound of garlic, to prevent and cure cancer in rodent tumor models (3,4,5,7,8,9,

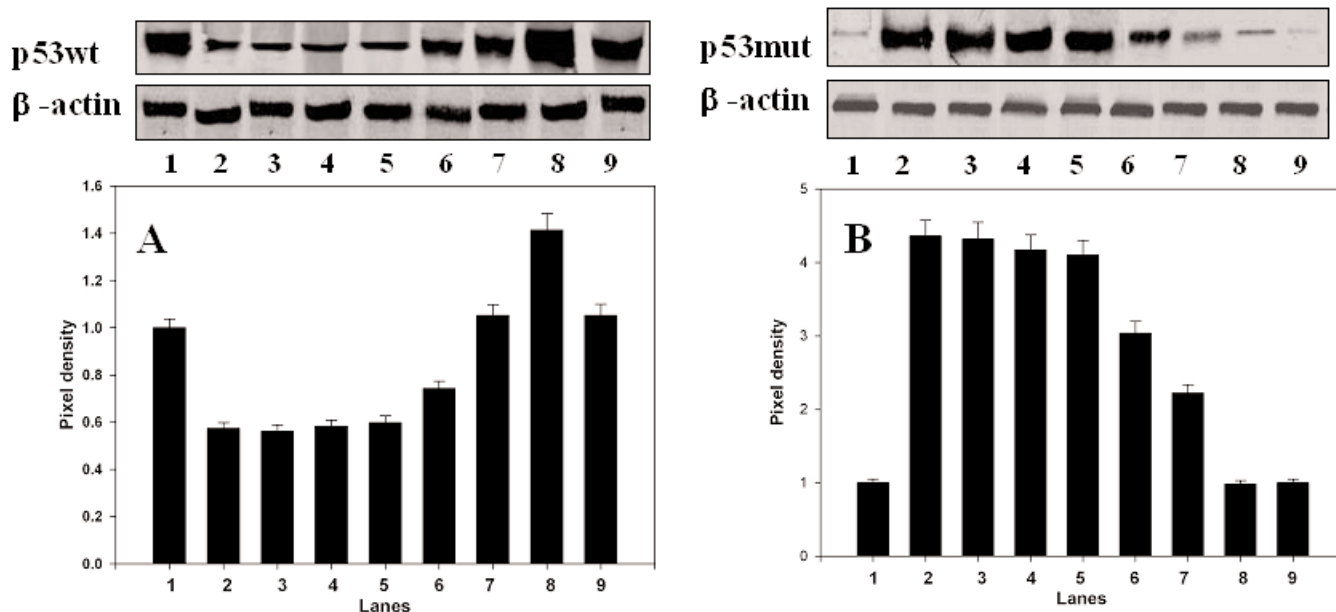


Figure 5. A. Effect of various formulations of DAS on the expression of p53wt in mouse skin tumors. Skin/tumor lysates were prepared as described in Materials and Methods. Lysates were resolved by electrophoresis and analyzed for p53wt using anti-p53wt antibodies. To quantify equal loading, membranes were re-probed with β -actin antibody. The intensity of the bands was quantitated using Image Analysis software on an Image Gel Documentation System. 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. B. Effect of various formulations of DAS on the expression of p53mut in mouse skin tumors. Skin/tumor lysates were prepared as described in Materials and Methods and analyzed for p53mut expression by Western blot analysis using antibody to p53mut. To quantify equal loading, membranes were re-probed with β -actin antibody. The intensity of the bands was quantitated using Image Analysis software on an Image Gel Documentation System. 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.

10,14,36,37). 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 has not allowed attainment of effective therapeutic concentration at the tumor site. Earlier studies showed that localized drug delivery to the site of skin tumor could be enhanced significantly through the use of liposome-based drug carriers (19,21,22). The present study focuses on an 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 among other factors. Lip-DAS liposomes showed an efficient (84 percent) suppression of tumor growth, while its pH-sensitive liposomal formulation was found to be more effective and induced around 94 percent tumor suppression (in comparison to the untreated control group). This clearly suggests that liposomization offers a new and effective option to increase the chemo-preventive

and anticarcinogenic potential of DAS in cancer therapy. The increased efficacy of liposomized DAS could be attributed to the fact that liposomes act as a sustained release system allowing greater accumulation of drug molecules at the tumor site than that achieved by its free form (29,38,39,40).

The higher efficacy of pH-Lip-DAS over Lip-DAS could be attributed to the fact that 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 the desired anticancer activity. In fact, pH sensitive liposomes undergo phosphatidyl-ethanolamine-mediated phase transition at acidic pH,

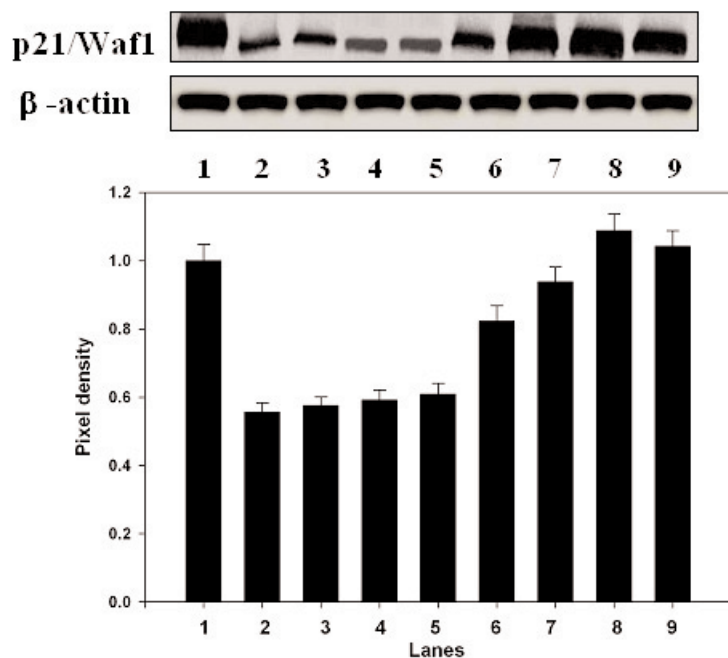


Figure 6. Effect of various formulations of DAS on the expression of p21/Waf1 in mouse skin tumors. Skin/tumor lysates were prepared as described in Materials and Methods and proteins samples from various groups were employed for Western blots. The level of p21/waf1 was assessed by Western blotting using anti-p21/Waf1 antibodies. To quantify equal loading, membranes were reprobbed with β -actin antibody. The intensity of the bands was quantitated using Image Analysis software on an Image Gel Documentation System. 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.

thereby delivering their content to the cytosol of the tumor cells (phosphatidylethanolamine is the main constituent of these liposomes). The polar head group of PE gets less hydrated as compared with the 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 PE not only facilitates the close approach of bilayers, it may also be involved directly in the merging process. In this context, PE can form the hexagonal H_{II} phase, the formation of which involves the development of a 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 (41,42). In addition, 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 (43,44,45), 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 (α -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 (46). It has been reported that polyunsaturated phosphatidylcholine (PC) and phosphatidylserine (PS) induce growth inhibition, differentiation and apoptosis in Caco-2 cells (47). 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 the intrinsic anticancer effect of DAS, presence of unsaturated fatty acids in liposomes also could be considered as a potential component to enhance the anticancer properties of the 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 the amount of the anticancer fatty acids used in the present study never touched the optimum threshold levels required for their anticancer effect as opposed to the effects possible had we used phospholipids that 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 candidates for use as drug delivery systems against cancer, including skin papilloma.

In the next phase of study, we deliberated the effect of liposomized 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 (48,49). We have reported earlier that DAS mediated modulation of p53 in DMBA induced skin tumors in Swiss albino mice (9). The importance of the p53

gene can be drawn from the fact that this gene is mutated in ~80 percent of all human malignancies (50). Because of its role in the regulation of the 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 (51). Several nuclear localization signals in the C-terminus of p53 facilitate its transport to the nucleus where it is required in the 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 its degradation (52). 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 (53). Taking the above 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 (52,54,55). In tumors, loss of p53wt prevents the activation of this growth control pathway (56). The failure to induce transcriptionally active p53wt plays a role in the unregulated growth of the tumors (57). 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 (58,59). 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 liposomization in neutral as well as pH-sensitive liposomes.

The upregulation of p53wt by chemo-preventive agents also is responsible for the transcriptional induction of p21/

Waf1 by directly interacting with its regulatory elements (57). As evident from the present study, liposomization of DAS in egg-PC or pH-sensitive liposomes ensued in upregulation of p21/Waf 1 as compared with the free form of the drug. The effect is more prominent in pH-sensitive liposomes as compared with 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 (60). 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 (61).

The observed better efficacy of liposomized 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 the problem of higher elimination of the drug from tumors, but the ability of liposomes to accommodate several thousand molecules in a single vesicle entity also helps in maintaining the effective drug concentration at the tumor site.

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REFERENCES

- Block E. (1985) The chemistry of garlic and onions. *Sci. Am.* 252:114-9.
- Agarwal KC. (1996) Therapeutic actions of garlic constituents. *Med. Res. Rev.* 16:111-24.
- Arora A, Kalra N, Shukla Y. (2006) Regulation of p21/ras protein expression by diallyl sulfide in DMBA induced neoplastic changes in mouse skin. *Cancer Lett.* 242:28-36.
- Prasad S, Kalra N, Shukla Y. (2006) Modulatory effects of diallyl sulfide against testosterone-induced oxidative stress in Swiss albino mice. *Asian J. Androl.* 8:719-23.
- Haber-Mignard D, Suschetet M, Berges R, Astorg P, Siess MH. (1996) Inhibition of aflatoxin B1 and N-nitrosodiethylamine induced liver preneoplastic foci in rats fed naturally occurring allyl sulfides. *Nutr. Cancer.* 25:61-70.
- Wargovich MJ, Imada O, Stephens LC. (1992) Initiation and post-initiation chemo-preventive effects of diallyl sulfide in esophageal carcinogenesis. *Cancer Lett.* 64:39-42.
- Wargovich MJ, Woods C, Eng VW, Stephens LC, Gray K. (1988) Chemoprevention of N-nitrosomethylbenzylamine-induced esophageal cancer in rats by the naturally occurring thioether, diallyl sulfide. *Cancer Res.* 48:6872-5.
- Sparmins VL, Barany G, Watterberg LW. (1988) Effects of organosulphur compounds from garlic and onions on benzo (a) pyrene induced neoplasia and glutathione S-transferase activity in the mouse. *Carcinogenesis.* 9:131-4.
- Arora A, Siddiqui IA, Shukla Y. (2004) Modulation of p53 in 7,12-dimethylbenz [a] anthracene-induced skin tumors by diallyl sulfide in Swiss albino mice. *Mol. Cancer Ther.* 3:1459-66.
- Singh A, Shukla Y. (1999) Antitumor activity of diallyl sulfide in two stage mouse skin model of carcinogenesis. *Biomed. Environ. Sci.* 11:258-63.
- Wargovich MJ. (1987) Diallyl sulfide, a flavor component of garlic (*Allium sativum*) inhibits dimethylhydrazine induced colon cancer. *Carcinogenesis* 8:487-9.
- Shukla Y, Arora A, Taneja P. (2003) Antigenotoxic potential of certain dietary constituents. *Teratog. Carcinog. Mutagen* 1:323-35.
- Smith TJ, Yang CS. (2000) Effect of organosulfur compounds from garlic and cruciferous vegetables on drug metabolism enzymes. *Drug Metabol. Drug Interact.* 17:23-49.
- Yang CS, Chhabra SK, Hong JY, Smith TJ. (2001) Mechanisms of inhibition of chemical toxicity and carcinogenesis by diallyl sulfide (DAS) and related compounds from garlic. *J. Nutr.* 131:1041-57.
- Guyonnet D, Siess MH, Le Bon AM, Suschetet M. (1999) Modulation of phase II enzymes by organosulfur compounds from Allium vegetables in rat tissues. *Toxicol. Appl. Pharmacol.* 154:50-8.
- Spiclin P, Homar M, Zupancic-Valant, Gasperlin M. (2003) Sodium ascorbyl phosphate in topical microemulsions. *Int. Pharm.* 256:65-73.
- Jia-You Fang, Tsong-Long Hwang, Yen-Ling

- Huanga, Chia-Lang Fang. (2006) Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. *Int. J. Pharm.* 310:131–8.
18. Muller RH, Radtke M, Wissing SA. (2002) Solid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv. Drug Deliv. Rev.* 54:131–55.
 19. Betz G, Aeppli A, Menshutina N, Leuenberger H. (2005) In vivo comparison of various liposome formulations for cosmetic application. *Int. J. Pharm.* 296:44–54.
 20. Kirjavainen M, Urtti A, Valjakka-Koskela R, Kiesvaara J, Mönkkönen J. (1999) Liposome-skin interactions and their effects on the skin permeation of drugs. *Eur. J. Pharm. Sci.* 7:279–86.
 21. Maestrelli F, Gonzalez-Rodriguez ML, Rasco AM, Mura P. (2005) Preparation and characterization of liposomes encapsulating ketoprofen-cyclodextrin complexes for transdermal drug delivery. *Int. J. Pharm.* 298:55–67.
 22. Cevc G. (1996) Transferosomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration and transdermal drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 13:257–388.
 23. Sharma A, Straubinger RM. (1994) Novel taxol formulations: Preparation and characterization of taxol-containing liposomes. *Pharm. Res.* 11:889–96.
 24. Skalko N, Peschka R, Atenschmidt U, Lung A, Schubert R. (1998) pH-sensitive liposomes for receptor-mediated delivery to chicken hepatoma (LMH) cells. *FERS.* 434:351–6.
 25. Bhatia A, Kumar R, Katare OP. (2004) Tamoxifen in topical liposomes: development, characterization and in-vitro evaluation. *J. Pharm. Pharm. Sci.* 7:252–9.
 26. Drummond DC, Meyer OM, Hong K, Kirpotin D, Papahadjopoulos D. (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.* 51:691–743.
 27. Guerin MR. (1978) Energy sources of polycyclic aromatic hydrocarbons. In: Gelboin HV, Tso POP, editors. *Polycyclic hydrocarbons in cancers*. vol 1, part I. New York: Academic Press. p.3–42.
 28. Dipple A, Mosschel RC, Bigger CAH. (1984) Polynuclear aromatic carcinogens. In: Charles E. Searle, editor. *Chemical Carcinogens*, vol 1. ACS Monograph 182. Washington, DC: American Chemical Society. p.41–126.
 29. Harasym TO, Cullis PR, Bally MB. (1997) Intratumor distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes. *Cancer Chemother. Pharmacol.* 40:309–17.
 30. Singleton WS, Gray MS and Brown ML. (1965) A method for adsorbent fractionation of cottonseed oil for experimental intravenous fat emulsions. *J. Am. Oil Chem. Soc.* 42:53–6.
 31. Owais M, Krishnakumar B, Jain RK, Bachhawat BK, C.M. Gupta. (1993) Tuftsin bearing liposomes as drug vehicles in the treatment of experimental aspergillosis. *FEBS Lett.* 326:56–8.
 32. Khan MA, Syed FM, Nasti HT, Saima Dagger K, Haq W, Shehbaz A, Owais M. Use of tuftsin bearing nystatin liposomes against an isolate of *Candida albicans* showing less in vivo susceptibility to amphotericin B. *J Drug Target* 2003;11(2):93-9.
 33. Serpi R, Piispala J, Jarvilehto M, Vahakangas K. (1999) Thapsigargin has similar effect on p53 protein response to benzo(a)pyrene DNA adducts as TPA in mouse skin. *Carcinogenesis.* 20:1755–60.
 34. Towbin H, Staehelin T, Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350–4.
 35. Lowry OH, Rosenbrough NK, Farr AL, Randall RJ. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193:265–75.
 36. Pinto JT, Rivlin RS. (2001) Antiproliferative effects of Allium derivatives from garlic. *J. Nutr.* 131:1058–60.
 37. Singh A, Shukla Y. (1998) Antitumor activity of diallyl sulfide on polycyclic aromatic hydrocarbon induced mouse skin carcinogenesis. *Cancer Lett.* 131:209–14.
 38. Fang JY, Hung CF, Hwang TL, Huang YL. (2005) Physicochemical characteristics and in vivo deposition of liposome-encapsulated tea catechins by topical and intratumor administrations. *J. Drug Target.* 13:19–27.
 39. Dayan N, Touitou E. (2000) Carriers for skin delivery of trihexyphenidyl HCl: ethosomes vs. liposomes. *Biomaterials.* 21:1879–85.
 40. El Maghraby GMM, Williams AC, Barry BW. (2001) Skin delivery of 5-fluorouracil from ultra-deformable and standard liposomes in vitro. *J. Pharm. Pharmacol.* 53:1069–77.
 41. Sergio Simoes, Vladimir Slepshkin, Nejat Duzgunes, Maria C. Pedroso de Lima. (2001) On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes. *Biochimica et Biophysica Acta* 1515:23–37.
 42. Yatvin MB, Kruetz W, Horwitz BA, Shinitzky M. (1980) pH-sensitive liposomes: possible clinical implications. *Science.* 210:1253–5.
 43. Huang SK et al. (1992) Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. *Cancer Res.* 52:6774–81.
 44. Dellian M, Helmlinger G, Yuan F, Jain RK. (1996) Fluorescence ratio imaging of interstitial pH in solid tumours: effect of glucose on spatial and temporal gradients. *Br. J. Cancer.* 74:1206–15.
 45. Helmlinger G, Yuan F, Dellian M, Jain RK. (1997) Interstitial pH and pO₂ gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nature Med.* 3:177–82.
 46. Jensi LJ, Zerouga M, Stillwell W. (1995) Omega-3 fatty acid-containing liposomes in cancer therapy. *Experimental Biology and Medicine* 210:227–233.
 47. Hossain Z, Konishi M, Hosokawa M, Takahashi K. (2006) Effect of polyunsaturated fatty acid-enriched phosphatidylcholine and phosphatidylserine on butyrate-induced growth inhibition, differentiation and apoptosis in Caco-2 cells. *Cell Biochem. Funct.* 24:159–65.
 48. Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. (1998) The p53 network. *J. Biol. Chem.* 273:1–4.
 49. Mowat MR. (1998) p53 in tumor progression: life, death and everything. *Adv. Cancer Res.* 74:25–48.
 50. Hollstein M, Sidransky D, Vogelstein B, Harris CC. (1991) p53 mutations in human cancers. *Science.* 253:49–53.
 51. Vousden KH, Lu X. (2002) Live or let die: the cell's response to p53. *Nat. Rev. Cancer* 2:594–604.
 52. Stommel JM et al. (1999) A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J.* 18:1660–72.
 53. Lane DP. (1992) p53, guardian of the genome. *Nature.* 358:15–6.
 54. Sengupta S, Vonesch JL, Waltzinger C, Zheng H, Waslyck B. (2000) Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. *EMBO J.* 19:6051–64.
 55. Lu W et al. (2000) Nuclear exclusion of p53 in a subset of tumors requires MDM2 function. *Oncogene.* 19:232–40.
 56. Burns PA et al. (1991) Loss of heterozygosity and mutational alterations of the p53 gene in skin tumors of interspecific hybrid mice. *Oncogene.* 6:2363–9.
 57. el-Deiry WS et al. (1994) WAF1/CIP1 is induced in p53 mediated G1 arrest and apoptosis. *Cancer Res.* 54:1169–74.
 58. Schwartz D, Goldfinger N, Kam Z, Rotter V. p53 controls low DNA damage-dependent premeiotic checkpoint and facilitates DNA repair during spermatogenesis. *Cell Growth Differ.* 1999; 10(10):665–75.
 59. Schwartz J, Shklar G, Trickler D. (1993) p53 in the anticancer mechanism of vitamin E. *Eur. J. Cancer B. Oral Oncol.* 29B:313–8.
 60. Yechiel E, Coste R. (2005) *From Ancient Potions To Modern Lotions: A Technology Overview and Introduction to Topical Delivery Systems.* Delivery System Handbook for Personal Care and Cosmetic Products: Technology, Applications and Formulations. Noyes Publications. Ed. M. R.
 61. Padamwar MN, Pokharkar VB. (2006) Development of vitamin loaded topical liposomal formulation using factorial design approach: Drug deposition and stability. *Int. J. Pharm.* 320:37–44.