Topical delivery of DNA oligonucleotide to induce p53 generation in the skin via thymidine dinucleotide (pTT)-encapsulated liposomal carrier

Yi-Ping Fang
Department of Biotechnology, Yuanpei University, Hsinchu, Taiwan

Introduction: Transcription factor p53 has a powerful tumor suppressing function that is associated with many cancers. Since the molecular weight of p53 is 53 kDa, it is difficult to transport across cell membranes. Thymidine dinucleotide (pTT) is an oligonucleotide that can activate the p53 transcription factor and trigger the signal transduction cascade. However, the negative charge and high water solubility of pTT limit its transport through cellular membranes, thereby preventing it from reaching its target in the nucleus. A suitable delivery carrier for pTT is currently not available.

Objective: The purpose of this study was to employ a nanoscale liposomal carrier to resolve the delivery problem, and increase the bioavailability and efficiency of pTT.

Methodology: The approach was to employ liposomes to deliver pTT and then evaluate the particle size and zeta potential by laser light scattering (LLS), and permeation properties of pTT in vitro in a Franz diffusion assembly, and in vivo in a murine model using confocal laser scanning microscopy (CLSM).

Results: We found that dioleoylphosphatidylethanolamine (DOPE) combined with cholesterol 3 sulfate (C3S) were the best ingredients to achieve an average desired vehicle size of 133.6 ± 2.8 nm, a polydispersity index (PDI, representing the distribution of particle sizes) of 0.437, and a zeta potential of −93.3 ± 1.88. An in vitro penetration study showed that the liposomal carrier was superior to the free form of pTT at 2–24 hours. CLSM study observed that the penetration depth of pTT reached the upper epidermis and potential of penetration maintained up to 24 hours.

Conclusion: These preliminary data demonstrate that nanosized DOPE/C3S liposomes can be exploited as a potential carrier of drugs for topical use in treating skin diseases.

Keywords: thymidine dinucleotide, p53, liposome, permeation ability, confocal laser scanning microscopy

Introduction

The p53 tumor suppressor gene is well known and provides a major anticancer defense mechanism. Almost 80% of human cancers are associated with mutations of the p53 gene, which selectively delete or silence p53, thus promoting tumor progression and metastasis.1,2 Normally, there are two classes of genes: oncogenes and tumor suppressor genes. One composed of oncogenes which cause the cell to divide in an unregulated manner when a single altered copy leads to mutation, and another composed of anti-oncogenes which provide a protection mechanism, also called a tumor-suppressor gene. Autoregulatory feedback mechanisms exist to achieve a balance between oncogenes and anti-oncogenes. In brief, the p53 gene acts as a regulator of anti-oncogenes or as a tumor suppressor due to its ability to maintain normal growth
control and genomic stability.\(^1\) Recent work showed that p53 is activated by multiple pathways, such as DNA damage, and oncogenic and oxidative stress; so different stress signals can mediate different signaling pathways.\(^4\)

Several skin cancers, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are also related to p53 mutations, and the site of the mutation is the basis for distinguishing skin cancers.\(^5;6\) Skin cancer induced by ultraviolet (UV) radiation may trigger apoptosis in a p53-dependent manner and the formation of sunburned cells (apoptotic keratinocytes). Numerous studies also indicated that triggering p53 induction inactivates the division of cancer cells.\(^7;8\) Thymidine dinucleotide (pTT), a fragment of an oligonucleotide, activates the p53 transcription factor and p53 protein, and triggers a cascade of DNA-repair enzymes according to in vitro and in vivo evidence which focuses on UV radiation-induced mutagenesis and carcinogenesis.\(^9\) Typically, oligonucleotides have a high molecular weight, a negative charge, and high water solubility, which causes problems when transporting them through cellular membranes to generate specific reactions in nuclei.\(^10\) According to the above data, the bioavailability of pTT is low when it is delivered through cellular membranes by passive diffusion.

Drug delivery systems were therefore designed to protect nucleic acids from being destroyed in environmental conditions and allow them to enter skin cells and reach the site of action. In designing formulation systems, biocompatibility is one of the most important considerations. Previous studies employed a mixed solution of 75% propylene glycol and 25% dimethyl sulfoxide (DMSO) to deliver a biologically active concentration of pTT to guinea pig skin, and despite its effectiveness, it did cause skin damage.\(^11;12\) Biocompatibility and safety must be considered to make such solutions feasible in clinical applications. Liposome systems are exploited as biocompatible delivery vesicles for both systemic and topical administration in clinical usage. Liposomal delivery systems can enhance the permeability of hydrophilic drugs and localize them to target tissues.\(^13\) Moreover, phospholipids are nontoxic and biodegradable, and prolong the half-life of drugs to attain a sustained-release effect. Previous studies demonstrated that phospholipids exhibit their enhancing effect on the application of topical delivery.\(^14;16\) Phospholipids are recognized to have skin permeation enhancing ability.

The applicability of liposomal carriers was demonstrated through extensive characterization of the particle size and zeta potential, and by transmission electron microscopic (TEM) studies. The cellular viability was evaluated using skin fibroblast and keratinocyte cell lines. Moreover, the skin delivery and penetration behavior of pTT-loaded liposomes were investigated by in vitro Franz diffusion cell and in vivo confocal laser scanning microscopy (CLSM).

Materials and methods

Materials

pTT with or without 5’ fluorescein was obtained from Protech Technology (Taipei, Taiwan). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, with a purity of >95%) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol-3-sulfate (C3S) was purchased from Sigma Chemical (St Louis, MO). Other chemicals used in the study were of reagent grade.

Preparation of liposomes

Liposomes were prepared according to the thin-film hydration method. DOPE and C3S were dissolved in 5 mL of a chloroform:methanol (2:1, v/v) solution in round-bottomed flasks. Organic solvent traces were evaporated by a rotary evaporator above the transition temperature of the lipid, and solvent traces were removed under a vacuum overnight. The thin film was fully hydrated with double-distilled water containing pTT at 100 µmol/L for 20 minutes. The vesicle suspension was dispersed by a probe-type sonicator (VCX 600; Sonics and Materials, Newtown, CT) at 25 W for 10 minutes.

Vesicle size and zeta potential

The vesicle size and zeta potential of the liposomes were measured by laser light scattering (LLS) with a helium-neon laser at 630 nm (Nano ZS1 90; Malvern, Worcestershire, UK). Liposomal suspensions were directly measured. The polydispersity index (PDI) was used to measure the size distribution. All vesicle sizes and zeta potentials were measured at 25°C. Measurements were repeated three times per sample for three samples.

Morphology observation by TEM

The liposomal vesicles were examined by TEM to characterize their microstructure. A drop of liposomes was applied to a carbon film-covered copper grid to form a thin-film specimen, which was stained with 1% phosphotungstic acid. The sample was then examined and photographed with a Jeol JEM-1230 TEM (Jeol Ltd, Tokyo, Japan).
Quantification of pTT-fluorescein
The pTT-fluorescein standard was determined spectrofluorometrically (Hitachi F-2500; Hitachi, Tokyo, Japan) at an excitation wavelength of 494 nm and emission at 519 nm. The calibration curve exhibited good linearity ($r = 0.97$).

Cellular viability
Cellular viability was used to determine the percentage cell survival by a 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, which produces formazan crystals in mitochondria within cells. A fibroblast cell line (HS68) and keratinocyte (HaCat) cells were seeded on 96-well plates at a cell density of $5 \times 10^4$ cells/well until cells were confluent. Dulbecco's modified Eagle's medium (DMEM) was replaced by liposomal suspensions for 12 and 24 hours at $37^\circ\text{C}$ in air containing 5% CO$_2$. We then added 50 µL of the MTT solution (50 µg/mL) to each well and incubated the wells for 4 hours. In order to dissolve the water-insoluble formazan, MTT/medium was removed from each well, and 100 µL of dimethyl sulfoxide (DMSO) was added to each one. The extent of cell survival was determined by an enzyme-linked immunosorbent assay (ELISA) reader at 550 nm.

In vitro evaluation of the skin penetration ability
We measured the topical delivery of pTT-fluorescein using a modified Franz vertical diffusion assembly (Ching Fa, HsinChu, Taiwan). Normal nude mouse skin was used as the barrier membrane. The donor medium consisted of 0.5 mL of a pTT-fluorescein liposomal formulation or control solution (pTT-fluorescein in distilled water), and the receptor medium consisted of 5.5 mL of pH 7.4 citrate-phosphate buffer. The available diffusion area was 0.785 cm$^2$. The stirring rate of the receptor medium consisted of 5.5 mL of pH 7.4 citrate-phosphate buffer. The available diffusion area was 0.785 cm$^2$. The stirring rate of the receptor medium was 600 rpm, and the temperature was maintained at $37^\circ\text{C}$. At appropriate intervals, 200 µL aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh buffer. The concentration of pTT-fluorescein was measured by a fluorescence spectrometer (F-2500, Hitachi, Tokyo, Japan) at $\lambda_{\text{excitation}}$ of 494 nm and $\lambda_{\text{emission}}$ of 519 nm. The cumulative amount of pTT-fluorescein was examined at the end of the in vitro experiments (24 hours) ($n = 4$).

In vivo topical delivery of the liposomal carriers
A glass cylinder with an available area of 0.785 cm$^2$ was placed with glue on the dorsal skin of a mouse. pTT-fluorescein that was or was not encapsulated in a liposomal formulation (100 µL) was added to each cylinder for 2, 4, 6, and 24 hours. At the end of the incubation period, the formulation was removed, and the skin was wiped ten times with a cotton cloth. The amount of pTT-fluorescein retained in the skin was measured by confocal laser scanning microscopy (CLSM). All procedures were carried out in the dark to prevent the influence of ambient light.

Evaluation of the penetration behavior by CLSM
CLSM was used to scan the pTT-fluorescein signal at different skin depths. The excised nude mouse skin was positioned on the microscopic slide with the stratum corneum (SC) side facing the cover glass. The CLSM Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used with an argon laser beam, excitation at 488 nm, and emission at 543 nm. Each skin sample was sliced in sections of 6–10 µm thick through the z-axis by CLSM. The pTT-fluorescein intensity and the permeated depth were detected by CLSM with Leica Confocal Software version 2.61 (Leica Microsystems, Wetzlar, Germany). Since CLSM was not able to be calibrated, we used arbitrary units to compare the data.

Statistical analysis
Data are expressed as the mean ± SD. Statistical analyses were performed using unpaired Student’s t-test with WINKS 6.0 software (Texasoft, TX). A 0.05 level of probability was used as the level of significance. Analysis of variance (ANOVA) was also used.

Results and discussion
The oligonucleotide fragment pTT can activate the p53 transcription factor and trigger tumor suppressor function. However, the negative charge and high water solubility of pTT limits its transport through cellular membranes and its ability to reach its target sites. Improving the poor bioavailability of pTT is necessary to proceed with the clinical investigations. The present study demonstrates that topical delivery of pTT using liposomal carriers can successfully resolve the delivery problem. We established the properties of pTT-loaded liposomes and their permeability behavior in vitro and in vivo models. CLSM study observed that the effect on skin reached the upper epidermis.

Characterization of pTT-loaded liposomes
The particle size, PDI, and zeta potential of the liposomes with and without pTT were measured by an LLS system, and the results are shown in Table 1. We observed no
distinct, undissolved crystals in the liposomal mixtures. The morphology of DOPE liposomes with pTT was also shown under TEM observations (Figure 1). DOPE liposomal vesicles were spherical. Moreover, results of the PDI were 0.39–0.44, which represent the well distribution of particle sizes. The composition with DOPE and C3S at a molar ratio 2.68 achieved the best condition. Liposome vesicles with or without pTT incorporation had particle sizes of 123 and 36 nm, respectively. Liposomes incorporating pTT were clearly larger than those without pTT (P < 0.05).

The skin penetration behavior was strongly dependent on the formulation characteristics. In our cases, diameters of the liposomal particles were controlled to <40 and 120 nm in empty and pTT-loaded liposomes, respectively. Generally, egg phosphatidylcholine (EPC) was used as phospholipid in the preparation of traditional liposome, the head group of phospholipid structure with choline N+(CH3)3 group. In this case, we chose phosphatidylethanolamine (PE) as the main phospholipid materials, with NH3+ as the head of the group. Past evidences pointed out that liposomes composed of EPC were significantly larger than those composed of PE in terms of particle size.17 This phenomenon was the same in our case. The PE type of phospholipid can form smaller particle sizes when incorporated with another surfactant in a proper ratio which is mainly related to the properties of PE. In addition, PE is a phospholipid which exhibits a high tendency to form the inverted hexagonal phase, particularly at an acidic pH.18 Protonation of the head group of PE, caused by a reduction of the pH in physiological conditions, neutralizes the negative charge, and the vesicles become destabilized as the PE component reverts to the hexagonal phase.19 Moreover, the zeta potentials of empty liposomes and pTT-loaded liposomes were about −63.17 ± 4.11 and −93.3 ± 1.88 mV, respectively. When pTT was loaded in liposomes the negative charge significantly increased, which was mainly attributed to pTT. The liposome charge plays a particular role in skin penetration, as discussed in the following section.

### Table 1 Physicochemical characteristics of liposomes with or without encapsulated pTT- fluorescein

<table>
<thead>
<tr>
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<th>Particle size (d.nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
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<tbody>
<tr>
<td>DOPE/cholesterol-3-sulfate (molar ratio = 2.68) empty</td>
<td>36.73 ± 0.61</td>
<td>0.39 ± 0.02</td>
<td>−63.17 ± 4.11</td>
</tr>
<tr>
<td>DOPE/cholesterol-3-sulfate (molar ratio = 2.68)+pTT- fluorescein</td>
<td>123.33 ± 3.91</td>
<td>0.44 ± 0.06</td>
<td>−93.3 ± 1.88</td>
</tr>
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</table>

**Note:** Data are presented as the mean ± SD (n = 3).

**Abbreviations:** PDI, polydispersity index; DOPE, dioleoylphosphatidylethanolamine; sD, standard deviation.

### Safety assessment

To apply liposomes as a pTT delivery carrier targeting the skin, acceptable toxicological properties should be assessed. We screened skin-associated cell lines including fibroblast and keratinocyte cells by using MTT assay. As shown in Figure 2, liposomes were examined compared to a negative control (pTT dissolved in double-distilled water) and a positive control (pTT dissolved in DMSO/PG: 7:3). There was no significant difference when liposomes were compared to the negative control group in fibroblast and keratinocyte cells (P > 0.05). Moreover, empty liposomes without pTT were also tested, and it was found that the vesicles themselves had no effect on cytotoxicity in the Hs68 cell line (P > 0.05; data not shown).

Past research attempted to utilize many approaches to transfer oligonucleotides into the skin. These approaches
Skin delivery ability by in vitro Franz diffusion penetration

Skin, the outermost superficial organ of the body, is an attractive target site for delivering/transferring drugs, active ingredients, and gene fragments. We evaluated the skin penetration ability of liposomal carriers to deliver pTT. The cumulative amounts (CAs) and flux of penetration were calculated from the slope after plotting the accumulated amount of pTT from each formulation as a function of time, as shown in Figure 3 and Table 2. The CA tendency decreased in the order of positive control > liposomes > negative control. Moreover, the CAs between the control group (consisting of a pTT aqueous solution) and liposomal carriers in nude mouse skin significantly differed (P < 0.05). In addition, significant improvements in pTT delivery in terms of the CA and flux were about 2.67- and 2.79-fold, respectively.

We also used a mathematical simulation to predict and more deeply understand the kinetics of the penetration mode. The model that best fitted the penetration data was selected based on the correlation coefficient (r) value in various models. Zero order was the (r) value between accumulative amounts versus time. First order was the (r) value between natural logarithm of accumulative amounts versus time. Higuchi mode was the (r) value between accumulative amounts versus square root of time. The in vitro penetration study found that pTT dissolved in the negative control solution still had low cumulative amounts which penetrated into the receptor compartment. This indicates that hydrophilic molecules may penetrate into hydrophobic skin in this case because their molecular weight was <500 kDa.27 However, the cumulative amounts did not greatly increase until 24 hours compared to

Figure 3 In vitro cumulative amount-time profiles of pTT-fluorescein in various vehicles across nude mouse skin.

Note: Data are presented as the mean ± SD (n = 4).

Abbreviation: SD, standard deviation.

included topical application, iontophoresis, electroporation, sonophoresis, laser, microdermabrasion, and gene guns.20-22 The results were effective and appeared promising for clinical application. Although the delivery efficiency showed improvement, some methods caused skin irritation.23 In the present study, we selected liposomes as the carrier for topical delivery. Liposomes were suggested to be suitable carriers for topical delivery since they are made with the same type of lipids as those present in the SC, and also the materials they are composed of are safe. In addition, the surfactants used to prepare the liposomes (most often phospholipids) are similar to those contained in the skin; liposomes easily mix with intercellular lipids of the SC. Phospholipids are not toxic; their incorporation in the SC layer changes the composition of the lipid mixture and may alter the permeability of the skin. Previous studies suggested the excellent ability of PE to facilitate drug or gene delivery into cells.24,25 In addition, some cases suggested that PE is easier to transfer across membranes of mammalian cells than phosphatidylcholine (PC) is.26
liposomes or the positive control group. The skin penetration model in the negative control group best fit the Higuchi order. The original data also showed a less-precise trend of the first and zero orders, for which the relative coefficients were 0.9325 and 0.9201, respectively. Accordingly, for pTT dissolved in the control solution, the penetration pattern followed Higuchi kinetics which means that the pTT concentration remaining in the receptor compartment within a period of time is limited due to the saturation capacity.

The positive control group presented high enhancement ratios in CAs and fluxes of about 4.98- and 4.21-fold compared to the negative control group; moreover, the penetration kinetics were also zero order. We divided the kinetics into two stages to discuss the kinetic modes. The initial state was from a period of 0–12 hours, in which pTT exhibited burst penetration; in the following period of 12–24 hours, it gradually achieved a steady-state. This indicates that the positive control group was saturated in the terminal period. On the other hand, in our case, enhancement ratios of CAs and flux in the first 24 hours were higher than those of the negative control group by about 2.67- and 2.79-fold. Moreover, the liposome system fit a zero order penetration model. Actually, the ideal delivery of drugs should follow zero order kinetics, wherein body circulation levels of drugs remain constant throughout the delivery period.

Skin penetration behavior by in vivo CLSM localization

We employed an in vivo CLSM technique to observe where pTT was localized in order to confirm and establish the true potential of the carrier. CLSM profiles were acquired after in vivo topical administration of the negative control, positive control, and liposomes on the back skin of nude mice for 24 hours. CLSM images suggested the accumulation, distribution, and skin penetration depth of pTT, as shown in Figure 4. The skin thickness was optically scanned at 10 µm increments for nine fragments from the surface of the skin (left to right). A cumulative xyz image was merged of all optical sections. A profile of skin depth versus fluorescence intensity was directly obtained from the Leica Confocal software. Accumulation of pTT-fluorescein localization was presented by time course after the in vivo topical administration for 2, 4, 6, and 24 hours (as shown in Figure 5A). According to the quantification data, all optical sections were merged, and the total intensities of pTT-fluorescein in nude mice skin (as shown in Figure 5B).

<p>| Table 2 In vitro permeation flux and cumulative amount of pTT across murine skin with negative control, positive control, and liposome treatment for 24 hours |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
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<tr>
<th></th>
<th>Flux (0–24 h)</th>
<th>Cumulative amount (CA)</th>
<th>ER (CA)</th>
<th>ER (flux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>5.11 ± 1.13</td>
<td>114.17 ± 15.70</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Positive control</td>
<td>21.97 ± 7.75</td>
<td>480.97 ± 172.04</td>
<td>4.21</td>
<td>4.98</td>
</tr>
<tr>
<td>Liposome</td>
<td>12.31 ± 1.63</td>
<td>304.38 ± 35.00</td>
<td>2.67</td>
<td>2.79</td>
</tr>
</tbody>
</table>

Note: Data are presented as the mean ± SD (n = 4).

Abbreviations: ER (the enhancement ratio) = (flux or CA of the liposome or positive control group/cumulative amount of the negative group).
The negative control group seemed to present few penetration trends after long-term application. The positive control group with in vivo application showed a great penetration flux of pTT, particularly after being applied for 4 hours; however, as the application time increased, the pTT intensity dramatically decreased. It tended to respond as the in vitro penetration study, and we assumed that was due to a saturation effect of the positive control vehicle. DMSO and PG are well known as potential penetration enhancers by affecting the intracellular lipids or the intracellular proteins of skin structure; hence, increasing the partitioning of the drug into stratum corneum. In our case, we truly observed an enhancing effect; but it only had a short-term influence. The phenomena assumed relevant with pTT rapid release from vehicle. Past studies pointed out that DMSO is a powerful penetration enhancer with rapid effects in both hydrophilic and lipophilic compounds. Caspers and his group indicated that DMSO remained within the tissue about 4 hours and a small fraction was detected for days, decreasing steadily with time by CLSM detection. In this case, DMSO has significantly effects in promoting permeation, however in the limitation period.

The liposome group with in vivo application also showed significantly increased pTT intensity, and the initial release of pTT from liposomes was low followed by sustained release from the carrier. We assumed that since the liposomal carrier was a mixture, we could not separate the entrapped and free drug parts in the experiment. From our
past evidence, phospholipid materials can assist hydrophilic ingredients penetrate across the skin in some circumstances. Additionally, active pharmaceutical ingredients and their polymorphic forms, crystallinity, particle size, solubility, and pharmaceutical dosage form can influence the release and penetration behaviors.

The results suggested that liposome particle sizes and physicochemical properties play particular roles in influencing pTT penetration into hydrophobic skin. The positive results could be expected, and were contributed by the small particle size and the lipophilic property which are well known from past evidence. Moreover, Carrer and his group pointed out that the presence of a negative charge in membranes of liposomes may allow for a better efficiency of penetration. The better penetration behavior was contributed by the lipid layer of the SC which contains a high ratio of negatively charged lipids. It is well known that the skin can act as a negatively charged membrane. Pharmaceutical formulations that most significantly improve drug absorption are made with soft (liquid) particles rather than with solid particles. Liposomes are themselves broken at the surface of skin and penetrate skin as free molecules together with the drug. As mentioned above, it is widely accepted that the skin penetration of organic particles is strongly dependent on particle sizes.

Additionally, the pTT-loaded liposomes showed a highly negative surface charge of about \(-93.3 \pm 1.88\) mV, which would be related to the penetration behavior. Regarding the properties of the skin, the lipid layer in the SC contains a high ratio of negatively charged lipids, and it is well known that the skin can act as a negatively charged membrane. In addition, several researchers reported that drug penetration can be influenced by modifying the surface charges of liposomes. Negatively charged vesicles generally produce a higher flux than their positively charged counterparts, which in turn can improve drug accumulation in the superficial skin strata.

In terms of the skin barrier, negatively charged and hydrophilic oligonucleotides cannot penetrate into the skin by passive diffusion. Hence, in order to become a useful therapeutic agent, effective delivery systems are required. Nanometer-size systems have garnered attention for delivering genes, antisense oligonucleotides, and small interfering RNA in recent years. In particular, vesicle size has a great influence on topical drug delivery systems; vesicles of <300 nm are able to deliver their contents into deeper layers of the skin to some extent. In the cases of liposomes with average diameters of <130 nm, there is the potential for delivery through the skin. In fact, particle sizes of between 100 nm and 1 µm are not nanoparticles, but are correctly defined as submicron particles which are no longer regarded as “suspect” (possibly hazardous). Hence, longer-term danger should not occur in that case.

### Conclusions

Choosing a suitable vehicle is one of the key factors in developing pharmaceutics and shortening the gap to proceeding with clinical applications. Past evidence showed that topical application enhanced the DNA repair capacity and protected skin from the carcinogenesis risk of solar UV irradiation by activating the tumor suppressor gene, p53. New insights bring an alternative strategy to reduce/protect against skin cancer risks. Formulating the drug dosage and establishing the carrier activity and permeation parameters are part of the final stage of progression to clinical use. Our study contributes to knowledge of the delivery system to increase actual applications and confirm the safety and utility of the carriers.

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### Disclosure

The author reports no conflicts of interest in this work.

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