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Transdermal Delivery of Paclitaxel-Anionic Nanoparticles to Epidermis Layer, Pterostilbene, and Pterostilbene glycoside, and Their Application for Treatment of Skin Cancer and Wrinkle

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A B S T R A C T

Composite nanoparticles, “anionic bicelles”, composed of anionic phospholipid of 1,2-dipalmitoyl- sn-glycero-3-phosphorylglycerol (DPPG) and paclitaxel were prepared by mixing them in water and a subsequent heating/cooling/ultrasonicating process. Anionic DPPG-paclitaxel nanoparticles (particle size: 12 nm) could be prepared after ultrasonic fragmentation at low temperature of 4°C. Upon addition of fluorescently labeled paclitaxel nanoparticles stabilized with DPPG to rat skin tissue (*in vitro*), the nanoparticles “anionic bicelles” (particle size: 12 nm) infiltrated into the epidermis layer penetrating stratum corneum (intercellular space: ca. 100 nm). In addition, during the anti-skin cancer test using mouse model of skin cancer, our study revealed that the numbers of papillomas of the mouse applied with paclitaxel nanoparticles stabilized with DPPG, “anionic bicelles”, to mouse skin were decreased, although those of the mouse applied with paclitaxel itself to mouse skin (control) were increased. Thus, this study established that since paclitaxel nanoparticles stabilized with DPPG could permeate stratum corneum and be incorporated into the epidermis layer of mouse, they could also treat skin cancer (*in vivo*). On the other hand, a mixture of anionic phospholipids, sodium cholate and Technol PG, allowed for formation of anionic nanoparticles, “anionic liposomes”, (particle size: 3 nm). This study established that as pterostilbene and pterostilbene glycoside could permeate stratum corneum and be incorporated into the epidermis layer, they could also treat wrinkle.

Introduction

Paclitaxel is a tricyclic diterpenoid compound naturally produced in the bark and needles of *Taxus brevifolia*. Because of its unique anticancer mechanism, it is already one of the most successful and widely used natural anticancer drugs. It is used in coronary heart disease, renal and hepatic fibrosis, inflammation, and axon regeneration (Gelmon, 1994; Luo *et al.,* 2017; Narayanan *et al.,* 2010; Perez, 2009; Uchida *et al.,* 2020; Uchida *et al.,* 2022; Zhu *et al.,* 2019). It possesses scientifically proven anticancer activity against ovarian, lung, and breast cancers. Skin is frequently exposed to oxidative stress from ultraviolet radiation, which presents a risk for the development of cancers such as melanoma, squamous cell carcinoma, and basal cell carcinoma. Efficient transdermal delivery of paclitaxel would be useful to cure these serious skin cancers. Skin tissue is composed of stratum corneum, epidermis, and dermis. However, the 10- to 40-µm-thickstratum corneum, consisting of densely packed cells, provides a barrier to protect the underlying tissue from infection, dehydration, chemicals, and mechanical stress. It is difficult to applicate paclitaxel for treatment of skin cancer, because it cannot penetrate the stratum corneum.

Phospholipids are promising molecules because they are synthesized in the body, and therefore, highly biocompatible. However, frequently utilized neutral phospholipids tend to form large-sized vesicles, which sometimes result in insufficient skin penetration. Attention is drawn on nanotechnology, the usefulness of nanoparticles containing paclitaxel, its opportunities, and also future perspective. Thus, preparation of phospholipid-based paclitaxel small-sized nanoparticles is still a challenging problem.

Skin aging is a multisystem degenerative process caused by several factors, such as, UV irradiation, stress, and smoke. Furthermore, wrinkle formation is a striking feature of photoaging and is associated with oxidative stress and inflammatory response. In the recent study, it was reported that caffeic acid, *S*-allyl cysteine, and uracil, which inhibit the degradation of type I procollagen, modulate UVB-induced wrinkle formation (Kim *et al.,* 2013).

Here, we report nanoparticles of paclitaxel and pterostilbene stabilized with anionic phospholipids of 1,2-dipalmitoyl-sn-glycero-3-phospho-1′-rac-glycerol (DPPG), “anionic bicelles”, or Technol PG, “anionic liposomes”. Also, their applications for treatment of skin cancer are reported. Additionally, we report the therapeutic effects of pterostilbene and pterostilbene glycoside for wrinkle.

Materials and Methods

General

Ultrasonication was performed by using a QSonica model ultrasonic homogenizer. The sizes of anionic nanoparticles were measured by using a Horiba model LA-960 laser diffraction particle size analyzer (SALD) or a Malvern model Zetasizer Nano ZSP zeta potential analyzer (DLS). DPPG was purchased from Avanti Polar Lipids.

Oregon Green-labelled paclitaxel was obtained as follows (Yanagi *et al.,* 2019). To a DMF solution of a mixture of paclitaxel and K_2CO_3, *N*-bocbromoethylamine was added, and the mixture was stirred under argon atmosphere at 50°C for 2 days. Then, the resultant mixture was extracted with EtOAc. The organic extract was evaporated to dryness under reduced pressure, and chromatographed on silica gel to allow the isolation of substituted compounds. The resulting compounds, dissolved in EtOAc, were treated with an excess of TFA, and the mixture was stirred overnight at room temperature. Then, aqueous solution of $NaHCO_3$ was slowly added to the reaction mixture, and the resultant mixture was extracted with EtOAc. The extract was evaporated to dryness under reduced pressure, and the counter ion was exchanged by chloride ion by adding HCl in dioxane and subsequently evaporated to obtain amine substituted paclitaxel. The mixture of amine substituted paclitaxel was precipitated in aqueous solution containing NaOH. The precipitate was dissolved in DMF, and subsequently, Oregon Green was added. After the mixture was stirred overnight at room temperature, the reaction mixture was slowly added to EtOAc. The precipitate formed was washed with water to obtain Oregon Green-labelled paclitaxel.

Preparation of DPPG-paclitaxel and DPPG-fluorescent paclitaxel

Paclitaxel was mixed with DPPG powder (5.0 wt%) in water and sonicated for 2 minutes to disperse homogeneously, and then heated at 60°C for 15 minutes where the solution turned clear (Uchida *et al.,* 2020). The

resulting mixture (DPPG-paclitaxel) was kept stand at room temperature for 1 hour before use. DPPG-Oregon Green-labelled paclitaxel (DPPG-fluorescent paclitaxel) was prepared in the same method as DPPG-paclitaxel except for using Oregon Green-labelled paclitaxel instead of paclitaxel. To prepare small-sized DPPG-Oregon Green-labelled paclitaxel nanoparticles (DPPG-fluorescent paclitaxel nanoparticles), the samples were ultrasonicated at 50 W for 3 hours with keeping the temperature at 4°C.

For the preparation of Technol PG nanoparticle, Technol PG (5 wt%) and cannabidiol was mixed with cholic acid-based surfactants of SC (0.5-5 wt%), CA (5 wt%), or CHAPSO (5 wt%) in water and ultrasonicated at 4°C for 2 min.

Invitro transdermal delivery of paclitaxel (DPPG-fluorescent paclitaxel nanoparticles) to epidermis layer

In vitro skin permeation tests were performed using a vertical Franz diffusion cell with an effective diffusion area of 0.95 cm^2 (Uchida *et al.,* 2022). Skin tissues were obtained from the abdominal hair of rats. The subcutaneous fat and other extraneous tissues of rat skin were trimmed and removed. A piece of excised skin (area 3.14 cm^2) was mounted on the Franz diffusion cell with the stratum corneum facing the donor compartment, in which DPPG-fluorescent paclitaxel nanoparticles (DPPG-Oregon Green-labelled paclitaxel nanoparticles) located. One circular SS Nikasol or SS HGA patch (area 0.785 cm^2) was applied to the stratum corneum side of the skin. The receptor compartment was filled with 3 mL of water and maintained at 32°C using a circulating water bath stirred with magnetic bars. For microscopic observations, skin tissue was embedded into OCT compound, frozen, and cryosectioned.

Invivo transdermal delivery of paclitaxel (DPPG-paclitaxel nanoparticles) to skin cancer

All animals were housed individually in cages under specific pathogen-free conditions during the experiments. Age- and sex-matched mice were used for the experiments (Ishida *et al.,* 2020). 8-Week-old male C57BL mice were used. Skin tumors were induced by two-step application of DMBA and 12-*O*-tetradecanoylphorbol-13-acetate. First, 25 µg of DMBA in 100 µL of acetone was applied onto the shaved dorsal skin of the mice on day 7 (1 week). On day 0, topical application of 30 µg of 12-*O*-tetradecanoylphorbol-13-acetate in 100 µL of acetone was initiated and was continued for 20 weeks with a frequency of twice a week. Tumor development was monitored on a weekly basis and lesions greater than 2 mm in length were counted as positive. DPPG-paclitaxel (paclitaxel incorporated in "anionic bicelles" (0.2 g/kg)) was applied to the rostral part of the back of mice five times a week. In the control experiment, paclitaxel itself was administrated in the same method as DPPG-paclitaxel.

Collagen 4A inducible activity of pterostilbene and pterostilbene glycoside

EpiDermFT EFT-400 human skin culture cells (MatTek Corp., Ashland, MA, USA) were treated with pterostilbene or pterostilbene glucoside. The EpiDermFT EFT-400 human skin cell model was cultivated at 37°C in 10% CO_2 atmosphere for 24 h, and were then mixed with 100 µM pterostilbene or pterostilbene glucoside. After one week incubation with compounds, cells were lysed, sonicated, and analyzed with Western blot analysis using anti-collagen 4A antibody and anti-GAPDH antibody (standard) (Sigma-Aldrich, St. Louis, MO, USA). The Protein solution was fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes containing fractionated proteins were blotted with anti-collagen 4A antibody. Membranes were then blotted with horseradish peroxidase conjugated second antibody and the immunoreactive protein bands were visualized by enhanced chemiluminescence.

Results and Discussion

Preparation of anionic nanoparticles

The size of DPPG-paclitaxel nanoparticles can be tuned by an ultrasonic fragmentation for the preparation of small-sized nanoparticles, "anionic bicelles". When size-controlled nanoparticles composed of fluorescent Oregon Green-labelled paclitaxel (Fig. 1) stabilized with DPPG (DPPG-fluorescent paclitaxel) were added to rat skin tissue, fluorescent Oregon Green-labelled paclitaxel molecules infiltrated into epidermis layer penetrating stratum corneum. DPPG is an anionic phospholipid synthesized in physiological conditions. Anionically charged DPPG is known to control lung pressure and functions of mitochondria because of repulsive force.

Additionally, DPPG molecules are expected to form kinetically stable nanoparticles maintaining the assemblies in physiological conditions because bilayer melting temperature of DPPG is higher than body temperature. Particle size analysis by laser diffraction revealed that the size of the paclitaxel hardly decreased. When paclitaxel was mixed with DPPG, a highly transparent dispersion was observed after the preparation. Tuning particle size is important for designing drug delivery systems. Especially, small-sized nanoparticles are preferable for the transdermal drug delivery system.

For this purpose, we next tried to create small-sized DPPG-paclitaxel nanoparticles. When we performed an ultrasonication treatment to the sample for 3 hours, the anionic DPPG-paclitaxel nanoparticles were fractionated to 12 nm-sized nanoparticles as confirmed by a particle size distribution analysis (Fig.2). The size of DPPG-paclitaxel nanoparticles, "anionic bicelles", in this work was remarkedly small, which have been hard to achieve so far. On the other hand, the size of the particles became smaller to 3 nm after being dispersed with Technol PG and SC, confirming the creation of smaller Technol PG nanoparticles, "anionic liposomes".

Invitro transdermal delivery of paclitaxel to epidermis layer

In the present study investigated the skin permeability of anionic DPPG-paclitaxel nanoparticles, "anionic bicelles" (Uchida *et al.,* 2020). For the evaluation of skin permeation capability, we prepared small-sized DPPG-paclitaxel nanoparticles, "anionic bicelles". The small-sized DPPG-fluorescent paclitaxel nanoparticles were obtained in the same method as small-sized DPPG-paclitaxel nanoparticles and were incubated with rat skin tissue placed on Franz diffusion cells. We prepared a histological section of the skin sample and performed fluorescent microscopic observation. Surprisingly, strong fluorescence was successfully observed due to the penetration of fluorescent paclitaxel molecules not only to the stratum corneum but also to the epidermis layer (Fig.3B), as compared with the sample without DPPG-fluorescent paclitaxel (Fig. 3A). Although the molecular structure of fluorescent paclitaxel is not exactly as same as that of paclitaxel, we expected that anionic DPPG-paclitaxel nanoparticles, "anionic bicelles", would have rather high skin permeation capability because the molecular structure of paclitaxel is much smaller than that of fluorescent paclitaxel.

Invivo transdermal delivery of paclitaxel to skin cancer

Mice started to develop papillomas later than 10 weeks after initial 12-*O*-tetradecanoylphorbol-13-acetate treatment. At 14 weeks after initial 12-*O*-tetradecanoylphorbol-13-acetate treatment, mice developed three papillomas and were used for the in vivo transdermal delivery experiment. The numbers of papillomas in anionic DPPG-paclitaxel nanoparticles-treated mouse (paclitaxel incorporated in "anionic bicelles"-treated mouse) were decreased, although those in paclitaxel-treated mouse (control mouse) were increased (Fig.4). These observations would explain that anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in "anionic bicelles") may contribute aschemo-preventive and anti-skin cancer agents.

Collagen 4A inducible activity of pterostilbene and pterostilbene glycoside

The level of type ⅣA collagen (COL4A) in the cells of EpiDermFT EFT-400 human skin model treated with pterostilbene and pterostilbene glucoside, which had been prepared by glycosylation of pterostilbene, was examined. At one week after treatment of the cells of EpiDermFT EFT-400 human skin model with pterostilbene, the COL4A level was increased in the cells (Fig. 5). The COL4A level of pterostilbene-treated cells was 1.4 (control). The COL4A level of pterostilbene glucoside-treated cells was elevated in the cells (1.7) by ca. 1.2-fold, compared with that of control cells, probably due to the cytotoxicity of pterostilbene.

We have reported paclitaxel nanoparticles stabilized by anionic phospholipids of DPPG, "anionic bicelles". The anionic DPPG-paclitaxel nanoparticles can be easily prepared by heating followed by a cooling treatment to aqueous mixtures of DPPG and paclitaxel, and the nanoparticles can be fractionated by an ultrasonication treatment to prepare small-sized nanoparticles, "anionic bicelles", (particle size: 12 nm). When the small-sized DPPG-fluorescent paclitaxel nanoparticles (particle size: 12 nm) were added to rat skin tissue, they penetrated the skin barrier of stratum corneum (intercellular space: ca. 100 nm) being incorporated into epidermis layer.

Applications of paclitaxel, which has no skin permeability, to anti-skin cancer materials have been still challenging because of its difficulty in transdermal delivery.

Figure.1 Chemical structures of paclitaxel and Oregon Green-labelled paclitaxel (fluorescent paclitaxel).

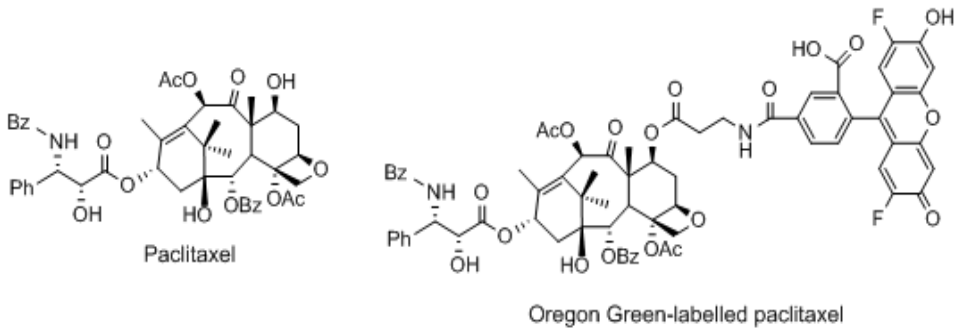


Figure.2 Particle size analysis of anionic DPPG nanoparticles, "anionic bicelles", prepared by a subsequent heating/cooling/ultrasonicating process.

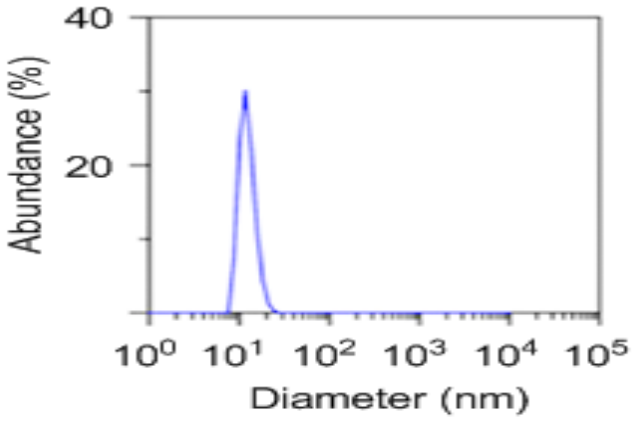


Figure.3 Fluorescent nanoscopic observation of skin sample (A) without and (B) with DPPG-fluorescent paclitaxel nanoparticles, "anionic bicelles".

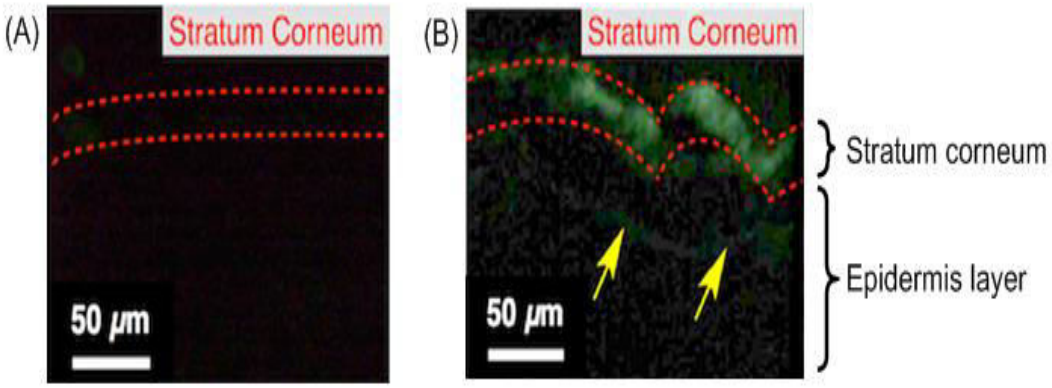


Figure.4 Numbers of papillomas of anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in "anionic bicelles")-treated mouse (●) and paclitaxel-treated mouse (control, □).

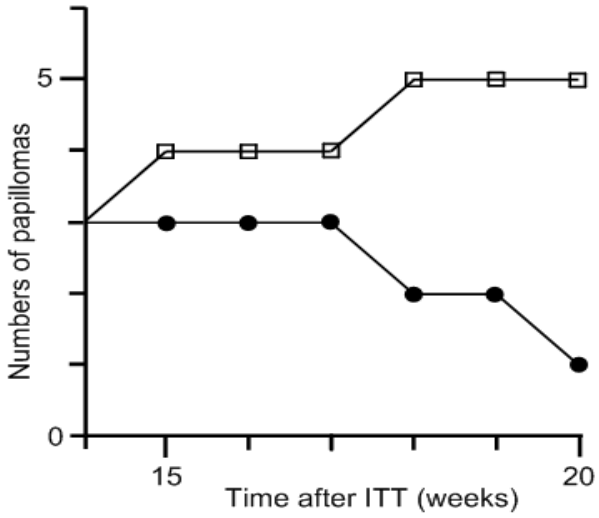
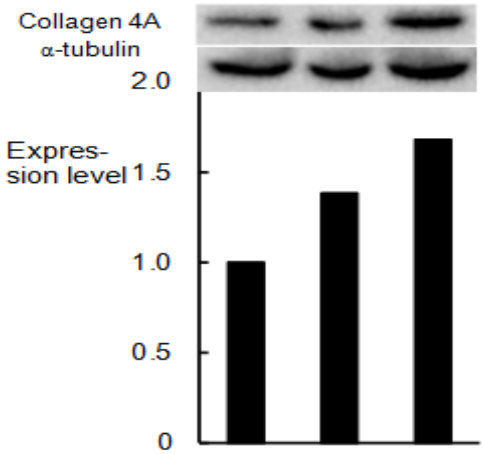


Figure.5 Effect of pterostilbene and pterostilbene glucosideon the expression of COL4Ain EFT-400 3D human skin cell model. A: Control, B: Pterostilbene, C: Pterostilbene glucoside.



The anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in "anionic bicelles") having skin permeability demonstrated in this study would be a new candidate as effective anti-skin cancer materials, which can infiltrate into epidermis layer decreasing numbers of papillomas. The effects of pterostilbene and pterostilbene glucoside on the EpiDermFT EFT-400 human skin model were examined. Both pterostilbene and pterostilbene glucoside, which had been prepared by glucosylation of pterostilbene, induced type Ⅳ A collagen (COL4A) expression in cells of EpiDermFT EFT-400 human skin model quickly. This is the first report on the induction of COL4A expression by plant polyphenols.

The COL4A level of pterostilbene glucoside-treated cells was higher than that of pterostilbene-treated cells. Stilbenes and, particularly, their glucosides may be useful for treatment of wrinkle. Further studies on the anti-skin cancer property of anionic DPPG-paclitaxel nanoparticles are now in progress in our laboratory.

Anionic DPPG nanoparticles, "anionic bicelles", were fractionated to 12 nm-sized nanoparticles after an ultrasonication treatment at 50 W for 3 hours with keeping low temperature at 4°C. Strong fluorescence was successfully observed due to the penetration of fluorescent paclitaxel molecules not only to the stratum corneum but also to the epidermis layer (B), indicating

that paclitaxel nanoparticles stabilized with DPPG, "anionic bicelles" (particle size: 12 nm), could permeate stratum corneum (intercellular space: ca. 100 nm) and be incorporated into the epidermis layer.

ITT: initial 12-*O*-tetradecanoylphorbol-13-acetate treatment. The numbers of papillomas in anionic DPPG-paclitaxel nanoparticles-treated mouse (paclitaxel incorporated in "anionic bicelles"-treated mouse) were decreased, although those in paclitaxel-treated mouse (control) were increased, suggesting that anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in "anionic bicelles") may contribute a schemo-preventive and anti-skin cancer agents, which can infiltrate into epidermis layer decreasing numbers of papillomas.

Author Contribution

Hiroki Hamada: Investigation, formal analysis, writing—original draft. Daisuke Uesugi: Validation, methodology, writing—reviewing. Kohji Ishihara:—Formal analysis, writing—review and editing. Ryusuke Hosoda: Investigation, writing—reviewing. Kei Shimoda: Resources, investigation writing—reviewing. Atsuhito Kuboki: Validation, formal analysis, writing—reviewing. Noriyuki Uchida: Conceptualization, methodology, data curation, supervision, writing—reviewing the final version of the manuscript. Yuya Kiriake: Investigation, formal analysis, writing—original draft.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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