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Preparation of Paclitaxel-Anionic Nanoparticles, Memantine (Memary) Gluco-oligosaccharides, α-Tocopherol Glycoside, Daidzein Glycoside, and Glycitein Glycoside and Their Application for Treatment of Skin Cancer, Dementia, Parkinson's Disease, and Allergy

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This study presents novel anionic bicelles—nanoparticles composed of DPPG and paclitaxel stabilized by cholic acid-based surfactants—prepared via a low-temperature (4°C) ultrasonic fragmentation process, yielding uniform 12-nm particles. These bicelles effectively penetrated the stratum corneum (intercellular spaces ~100 nm) and infiltrated the epidermis in vitro, demonstrating potent anti-skin cancer activity against A431, SCL I, and KB cells. *In vivo*, topical application significantly reduced papilloma formation in a murine skin cancer model, whereas free paclitaxel exacerbated tumor growth, confirming enhanced efficacy and targeted delivery. Concurrently, glycosylated memantine derivatives (memary-gluco-oligosaccharides) were enzymatically synthesized and exhibited superior neuroprotective effects: enhancing survival of TH^+ neurons in rat midbrain cultures and protecting hippocampal neurons against Aβ toxicity. *In vivo*, these conjugates crossed the blood-brain barrier following intraperitoneal or oral administration, increasing synaptic density in the dentate gyrus and improving spatial learning. In 6-OHDA-induced hemiparkinsonian mice, memary-gluco-oligosaccharides reduced ipsilateral rotations and restored contralateral hindlimb stepping, indicating marked motor symptom amelioration. Furthermore, α-tocopherol glucoside demonstrated potent anti-allergic activity against wheat allergens (gliadin/glutenin), while daidzein and glycitein glucosides effectively suppressed soybean allergen (globulin)-induced responses. Collectively, these findings establish that lipid-based nanocarriers enable transdermal drug delivery for skin cancer therapy, while enzymatic glycosylation enhances bioavailability, neuroprotection, and anti-allergic efficacy—offering promising platforms for treating neurological disorders and allergic conditions.

Introduction

Paclitaxel, which is a naturally produced compound in the bark and needles of *Taxus brevifolia*, is a tricyclic diterpenoid compound. Paclitaxel is already one of the most successful and widely used natural anticancer drugs, because of its unique anticancer mechanism. Paclitaxel is used for treatment of coronary heart disease, renal and hepatic fibrosis, inflammation, and axon regeneration (Gelmon, 1994; Narayanan *et al.,* 2010; Perez, 2009; Uchida *et al.,* 2020; Uchida *et al.,* 2022; Zhu *et al.,* 2019). It has scientifically proven anticancer activity toward 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-thick stratum 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 biologically friendly molecules to living body because they are synthesized in the body. Therefore, they are highly biocompatible.

However, frequently utilized neutral phospholipids tend to form large-sized vesicles, which sometimes result in insufficient skin penetration.

Nanotechnology has attracted biomedical attention for 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.

The blood–brain barrier (BBB) exists in the brain as a selective semipermeable border that prevents solutes in the circulating blood from non-selectively crossing the extracellular fluid of the central nervous system where neurons exist (Daneman *et al.,* 2015).

It comprises endothelial cells of the capillary wall, astrocyte end-feet ensheathing the capillary, and pericytes fixed firmly in the capillary basement membrane (Ballabh *et al.,* 2004). While the BBB system allows the passage of some small molecules by passive diffusion, it also permits the selective transport of various nutrients, ions, organic anions, and macromolecules, such as glucose and amino acids, crucial to neuronal functioning (Ballabh *et al.,* 2004).

Here, we report nanoparticles, “anionic bicelles”, of paclitaxel stabilized with anionic phospholipids of DPPG. Also, their applications for treatment of skin cancer are reported. Additionally, we report the therapeutic effects of memantine (memary) gluco-oligosaccharides, α-tocopherol glycosides, daidzein glycosides, and glycitein glycosides for dementia and allergy.

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).

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.

In vitro transdermal delivery of paclitaxel incorporated in “anionic bicelles” (anionic 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.

In vitro anti-cancer activity of paclitaxel incorporated in "anionic bicelles" (anionic DPPG-paclitaxel nanoparticles) against skin cancer A431 cells, SCL I cells, or KB cells

The sensitivity of A431 (SCL I, or KB) cells to paclitaxel incorporated in DPPG (paclitaxel incorporated in "anionic bicelles") was determined as follows. Cells were diluted with culture medium to the seeding density, suspended in 96-well tissue culture plates, preincubated at 37°C for 4 h, and then treated for 24 h with paclitaxel incorporated in DPPG at various concentrations. After incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole) solution was added to each well and the plates were further incubated for 4 h. Absorbance at 570 nm was measured with a microplate reader model 450 (BIO-RAD).

In vivo anti-cancer (anti-tumor) activity of paclitaxel incorporated in "anionic bicelles" (anionic DPPG-paclitaxel nanoparticles) against 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.

In vitro neuroprotective effect of memantine (memary) gluco-oligosaccharides on DA neurons in rat primary midbrain cultures

cDNA of glucosyltransferase from *P. americana* (*Pa*GT) was cloned into pQE30, and the resulting plasmids were transformed into *E.coli* M15 cells. The purified enzyme solution was dialyzed with 50 mM Tris-HCl (pH 7.2) containing 5 mM dithiothreitol, and stored at –80 °C. Glucosylation reactions were performed at 35 °C for 24 hours in 5 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with memantine (memary), UDP-glucose, and enzyme *Pa*GT. The incubation was stopped by adding 1.5% trifluoroacetic acid; the reaction mixture was analyzed by HPLC. The resulting memantine (memary) glucoside was applied for further glycosylation by CGTase to give memantine (memary) gluco-oligosaccharides.

In the presence of memantine (memary) gluco-oligosaccharides (0.1, 1, 3, 10 µM), DA neurons were recognized with the polyclonal antibody against TH in rat primary midbrain cultures, and microglia were detected with the OX-42 antibody against CR3 receptor, according to previously described protocol (Liu *et al.,* 2003). Nine representative areas per well of the 96-well plate were counted under the microscope at 100 magnifications, for visual counting of TH-positive neurons. Counting was performed in a double-blind manner by two individuals, and conclusions were drawn only when the difference was within 5%.

In vitro neuroprotective effect of memantine (memary) gluco-oligosaccharides on rat hippocampal neurons treated by Aβ

The effect of memantine (memary) gluco-oligosaccharides on rat hippocampal neurons treated by Aβ was investigated. Aβ was administrated to the culture of rat hippocampal neurons at a dose of 2 µM. The living

hippocampal neurons were treated with memantine (memary) gluco-oligosaccharides at a single dose of 50 μM for 24 h in this study. The number of apoptotic cells was determined by double staining with Hoechst 33258 and propidium iodide.

In vivo BBB penetrating ability of memantine (memary) gluco-oligosaccharides

The mice were orally injected once with memantine (memary) glucoside, memantine (memary) gluco-oligosaccharides, or memantine (memary) (control) to test their BBB penetration abilities. One hour later, they were sacrificed by cervical dislocation, after which their brain tissue samples were quickly processed by rinsing with cold sodium phosphate buffer, then frozen and stored at −20°C. Subsequently, curcumin was extracted, after which its concentration in the brain sample was determined using HPLC. Tissue samples were first homogenized in sodium acetate buffer, and tissue homogenates were ultrasonicated in 0.1% Triton X-100. Then, in a flask containing the homogenate mixture, α-glucosidase, β-glucosidase, and β-glucuronidase were added and incubated at 36°C for one hour. Organic compounds were finally extracted with ethyl acetate. After three extraction steps, ethyl acetate was evaporated. Samples were dissolved in methanol to give brain extracts sample. Finally, the extracted memantine (memary) was quantified by HPLC. The mice, which were intraperitoneally injected once with these compounds, were tested in the similar method with orally injected mice.

In vivo BBB penetration of memantine (memary) gluco-oligosaccharides and Y-maze test

A Y-maze with three arms was constructed with gray plastic, then it was equipped with a partition that isolates an arm. The experiment involved a 5-min trial 1, separated by a 40-min interval, followed by a 5-min trial 2. During the familiarization phase (trial 1), one arm (arm C: novel arm) of the Y-maze was closed with a partition. Then, while we placed one SAMP8 in one arm (arm A) of the two remaining arms (arms A and B) and the mouse allowed to explore the maze for five minutes, the partition was removed after a 40-min interval. Afterward, for five minutes, the mouse had free access to all three arms during the retrieval phase (trial 2). The time of the novel arm (arm C) exploration was only counted when the mouse put his hind feet in that arm. Then, the percentage of time spent in the novel arm C was calculated. Finally, memantine (memary) was orally injected every day for five days to mouse (one injection per day) (the control), whereas memantine (memary) gluco-oligosaccharides were orally injected every day for five days to mouse (one injection per day (200 mg/kg)) (the memantine (memary)-oligosaccharides-treated mouse). In case of intraperitoneal injection of the gluco-oligosaccharides to mice, injection and Y-maze test were demonstrated in the same method as described above.

In vivo BBB penetration of memantine (memary) gluco-oligosaccharides and novel object recognition test

The C57BL mouse, which was administered memantine gluco-oligosaccharides orally, was used in novel object recognition test. Memantine itself was administrated to the mouse. The C57BL mouse was first introduced to two identical sample objects for 20 min for free exploration. The objects were fixed to the cage bottom and placed in the cage at one end near the corners, so that the mouse was able to move around the objects. The time was taken with a stopwatch when the mouse was exploring an object, placing the nose within 2 cm of either object. Climbing or biting the objects were not counted as exploration. A copy of the sample object and a new object were placed, 3-4 h later, at one end of the cage as earlier. The 5-min test started when the mouse approached either object for the first time. The exploration time of each object and the novelty preference index (NPI) in % (time exploring the novel object×100/ total exploration time) were counted. Mice that explored the objects in the test less than 3 s were discarded. The C57BL mouse, which was injected memantine gluco-oligosaccharides or memantine itself intraperitoneally, was tested in the same manner as described above.

In vivo rotation test of memantine (memary) gluco-oligosaccharides

Drug-induced rotation was validated in the 6-OHDA-induced hemi-parkinsonism mice. Apomorphine-induced rotation test was performed to study the hypersensitivity of the lesioned striatum. Memantine (memary) was orally injected every day for five days to mouse (one injection per day). On the other hand, memantine (memary) gluco-oligosaccharides were orally injected every day for five days to mouse (one injection per day (10 mg/kg)) (the

memantine (memary)-oligosaccharides-treated mouse). In case of intraperitoneal injection of the gluco-oligosaccharides to mice, injection and test were demonstrated in the same method as described above. Mice were placed in a clear plexiglass cylinder and ipsilateral and contralateral turns were relative to the site of the lesion. Full turns were counted in the ipsilateral and contralateral directions during a 20 min window of peak rotational response and data are expressed as net rotations. As for 6-OHDA-induced hemi-parkinsonism mice, to which sample was administrated orally or intraperitoneally, total number of spontaneous turns and contralateral turns was counted. Turns in the ipsilateral directions were indicated as % of total turns.

In vivo hind limb steps test of memantine (memary) gluco-oligosaccharides

Drugs were injected to the 6-OHDA-induced hemi-parkinsonism mice orally or intraperitoneally. Memantine (memary) was orally injected every day for five days to mouse (one injection per day). On the other hand, memantine (memary) gluco-oligosaccharides were orally injected every day for five days to mouse (one injection per day (10 mg/kg)) (the memantine (memary)-oligosaccharides-treated mouse). In case of intraperitoneal injection of the gluco-oligosaccharides to mice, injection and test were demonstrated in the same method as described above. Total count of spontaneous hind limb steps and that of contralateral steps were investigated in a 5-min test. Steps of the contralateral hind limb were indicated as % of total counts.

Anti-allergic activity of α-tocopherol glycoside, and daidzein glycoside and glycitein glycoside against glutenin and globulin

Glucosylation reactions were performed at 35 °C for 24 hours in 5 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with α-tocopherol, daidzein, or glycitein, UDP-glucose, and enzyme *Pa*GT. When using amylase as a biocatalyst, the mixture of acetonitrile and water was used for a solvent. The incubation was stopped by adding 1.5% trifluoroacetic acid; the reaction mixture was analyzed by HPLC. The yield of the products was calculated on the basis of the peak area from HPLC using the calibration curves prepared by the HPLC analyses of authentic glycosides.

Effects of compounds on O_2^- generation from rat neutrophils were examined as follows. Male Wistar rats, each weighing 250 g, were used. Under ether anesthesia, whole blood was collected from the carotid artery and diluted twice with Hanks' balanced salt solution (HBSS). Neutrophils were purified by Percoll density gradient centrifugation. O_2^- generation from rat neutrophils was measured by the cypridina luciferin analog-dependent chemiluminescence. Neutrophil suspensions were incubated for 3 min in HBSS containing cypridina luciferin analog and sample at 37°C in the dark. Five seconds later, fMLP was added into the assay mixture. Cypridina luciferin analog-dependent chemiluminescence was monitored. The results are expressed in terms of the percentage reduction of the O_2^- generation from rat neutrophils at 5 min after the administration of fMLP by test compounds.

The effects of test compounds on compound 48/80-induced histamine release from rat peritoneal mast cells were examined as follows. Peritoneal mast cells were collected from the abdominal cavities of rats (Male Wistar rats, Nippon SLC) and purified to a level higher than 95%. The purified mast cells were suspended in a physiological buffered solution (PBS) containing NaCl, KCl, $CaCl_2$, glucose, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to give approximately 10^4 mast cells/mL. Cell viability was always greater than 90% as judged by the trypan blue exclusion test. Mast cells were preincubated with the test compound for 15 min at 37°C, and subsequently exposed to compound 48/80. Histamine release was determined by a fluorometric assay, and was expressed as a percentage of total histamine.

The inhibitory action of test compounds on IgE antibody formation was examined as follows. Glutenin, gliadin, or 7S-globulin was used as the antigen, and $Al(OH)_3$ and pertussis toxin were used as the adjuvants. Sensitization was made by injection of a mixture of the antigen and the adjuvant into the paws of each rat (male). Paw edema was measured 24 h after injection and the treated rats were divided in groups with an equal average swelling volume. Each sample was dissolved in physiological saline containing 10% Nikkol and the solution containing test compound was injected daily into each rat for 11 d starting on the day of grouping. Hydrocortisone was used as the positive control. The amount of IgE was measured on the 15th day. The results were expressed as plasma IgE levels.

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 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. 1). The size of DPPG-paclitaxel nanoparticles, "anionic bicelles", in this work was remarkedly small, which have been hard to achieve so far (Uchida *et al.,* 2020).

In vitro transdermal delivery of paclitaxel incorporated in "anionic bicelles" (anionic DPPG-fluorescent paclitaxel nanoparticles) to epidermis layer

We 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. 2B), as compared with the sample without DPPG-fluorescent paclitaxel (Fig. 2A). 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 (Uchida *et al.,* 2020).

In vitro anti-cancer activity of paclitaxel incorporated in "anionic bicelles" (anionic DPPG-paclitaxel nanoparticles) against skin cancer A431 cells, SCL I cells, or KB cells

The cytotoxic activity of paclitaxel incorporated in DPPG nanoparticles toward human A431 (or SCL I) cells was examined. A431 (or SCL I) cells were diluted, suspended in 96-well tissue culture plates, preincubated, and then treated with paclitaxel incorporated in DPPG nanoparticles. After incubation, MTT solution was added to each well and the plates were further incubated. Absorbance at 570 nm was measured. The cytotoxic activity of paclitaxel incorporated in DPPG nanoparticles (IC_{50}=22 μM for A431 cells, IC_{50}=20 μM for SCL I cells, IC_{50}=19 μM for KB cells) was higher than paclitaxel itself (control) (IC_{50}=30 μM for A431 cells, IC_{50}=33 μM for SCL I cells, IC_{50}=25 μM for KB cells).

In vivo anti-cancer (anti-tumor) activity of paclitaxel incorporated in "anionic bicelles" (anionic DPPG-paclitaxel nanoparticles) against 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) were increased (Fig. 3). These observations would explain that anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in

"anionic bicelles") may contribute as chemo-preventive and anti-skin cancer agents.

In vitro neuroprotective effect of memantine (memary) gluco-oligosaccharides on DA neurons in rat primary midbrain cultures

Incubation of glucosyltransferase from *Phytolacca americana* (*Pa*GT) with memantine (memary) gave glucoside as the sole product. Glucosylation of memantine (memary) with *Pa*GT described here is considerably efficient method to give memantine (memary) glucoside rather than chemical glucosylation. Biocatalytic glycosylation of memantine (memary) glucoside with CGTase was attempted to synthesize memantine (memary) gluco-oligosaccharides.

In the previous report, DA uptake assay was used as a functional index, and immuno-cytochemical staining for TH-positive (a marker for DA neurons) neurons was used for both morphometric analysis and cell count to assess the viability of DA neurons in rat primary midbrain neuron-glia cultures (Wu *et al.,* 2009). To cultures were added various concentrations of memantine (memary) gluco-oligosaccharides (0.1-30 μM) or vehicle seven days after seeding. DA uptake assay was performed one week later. Memantine (memary) gluco-oligosaccharides in the range of 3-10 μM enhanced the capacity of DA uptake in a dose-dependent manner (Wu *et al.,* 2009). That memantine (memary) gluco-oligosaccharides increased (0.1, 1, 3, and 10 μM) the number of TH-positive neurons (DA neurons) in a dose-related manner (310, 330, 525, and 790) was found by cell count analysis. These results indicated that memantine (memary) gluco-oligosaccharides enhanced survival of DA neurons in rat primary midbrain cultures. Memantine (memary) gluco-oligosaccharides showed high neuroprotection of DA neurons.

In vitro neuroprotective effect of memantine (memary) gluco-oligosaccharides on rat hippocampal neurons treated by Aβ

The effect of memantine (memary) gluco-oligosaccharides on the relative numbers of rat hippocampal neurons treated by Aβ was examined. The ratio of the relative numbers of cells with signs of apoptosis in the culture of rat hippocampal neurons with the addition of 2 μM Aβ and 50 μM memantine (memary) gluco-oligosaccharides was 8%. In case of addition of 2 μM Aβ to the culture of rat hippocampal neurons (control), the ratio of the numbers of apoptotic cells in rat hippocampal neurons was 25%. Memantine (memary) gluco-oligosaccharides showed strong neuroprotective effects toward Aβ treated rat hippocampal neurons.

In vivo BBB penetrating ability of memantine (memary) gluco-oligosaccharides

Memantine (memary) gluco-oligosaccharides were orally injected to the mouse. Mouse brain tissue samples were processed as described in the Materials and Methods. After homogenizing tissue samples in sodium acetate buffer, the homogenates were ultrasonicated and treated by hydrolysis with glycosidases. Afterward, the products were extracted with ethyl acetate to prepare brain extracts.

The obtained memantine (memary) was subsequently quantified by HPLC analysis of the brain extracts. Thus, memantine (memary) was detected. The HPLC analysis results of the brain extracts sample indicated that memantine (memary) gluco-oligosaccharides were incorporated into the mouse brain tissue. Investigations also revealed that the brain extracts sample of the mouse treated with memantine (memary) (control) contained no memantine (memary), indicating that it hardly migrated to the mouse brain tissue. These results suggest that memantine (memary) gluco-oligosaccharides, which were orally injected into mouse, could penetrate the BBB migrating to the mouse brain. In case of intraperitoneal injection with memantine (memary) gluco-oligosaccharides, curcumin was detected. The brain extracts sample of the mouse treated with memantine (memary) (control) contained no memantine (memary), suggesting that it hardly migrated to the mouse brain tissue. These investigations indicate that memantine (memary) gluco-oligosaccharides, which were intraperitoneally injected into mice, could smoothly penetrate the BBB in the mouse brain.

In vivo BBB penetration of memantine (memary) gluco-oligosaccharides and Y-maze test

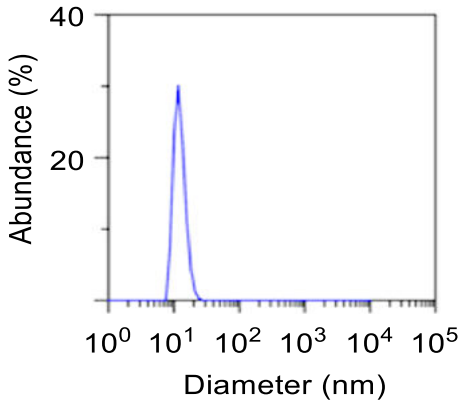
In the Y-maze test using SAMP8, time spent in the novel arm (arm C) by memantine (memary)-oral-administrated mouse, memantine (memary) gluco-oligosaccharide-oral-administrated mouse, memantine (memary)-intraperitoneal-administrated mouse, and memantine

(memary) gluco-oligosaccharide-intraperitoneal-administrated mouse were 102, 139, 100, and 125. The time spent in the novel arm of the Y-maze by the mouse intraperitoneally injected with memantine (memary) gluco-oligosaccharides, was longer than that spent by the control mouse, into which memantine (memary) alone was intraperitoneally injected. In case of Y-maze test using SAMP8 mouse orally injected with memantine (memary) gluco-oligosaccharides, the time spent in the novel arm of the Y-maze was longer than that spent by the control mouse. In both cases, the time spent in the novel arm by memantine (memary) gluco-oligosaccharides-treated mouse was higher than that of the time spent by the control mouse. These results suggest that memantine (memary) gluco-oligosaccharides penetrated the BBB and were incorporated into the brain tissue of SAMP8, enhancing spatial learning of the mouse.

In vivo BBB penetration of memantine (memary) gluco-oligosaccharides and novel object recognition test

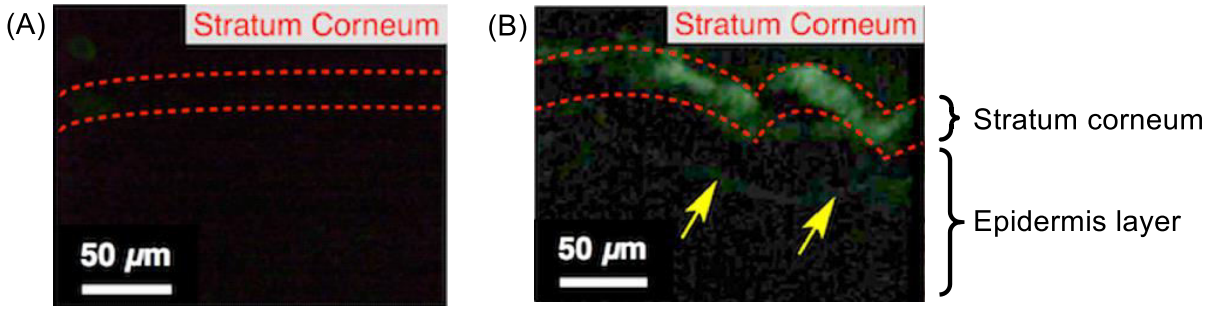
It is well known that the male mice use scent marking of their territory, including objects there. The substance for scent marking is long-lasting and insoluble to water.

Figure.1 Particle size analysis of anionic DPPG nanoparticles, "anionic bicelles", prepared by a subsequent heating/cooling/ultrasonicating process.



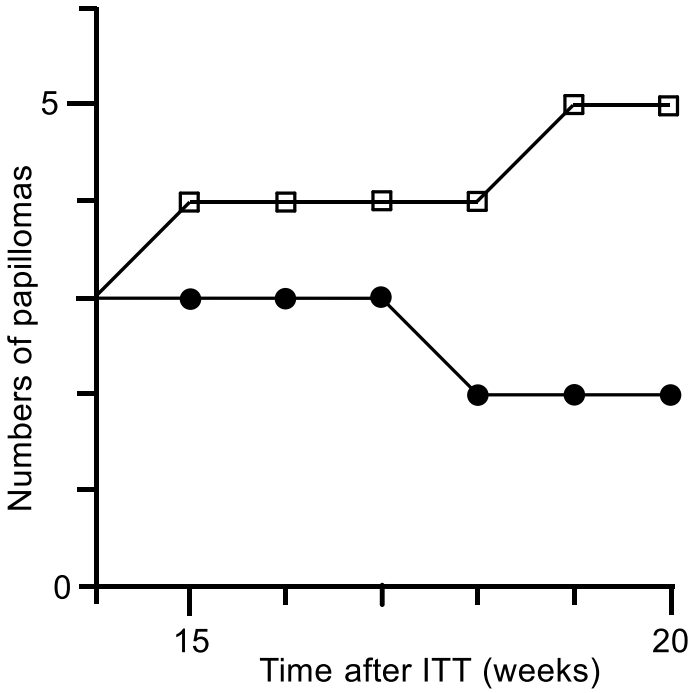
Anionic DPPG nanoparticles, "anionic bicelles", were fractionated to 12 nm-sized nanoparticles after an ultrasonication treatment at 50 W for 3 hours with keeping low temperature at 4°C.

Figure.2 Fluorescent nanoscopic observation of skin sample (A) without and (B) with DPPG-fluorescent paclitaxel nanoparticles, "anionic bicelles".



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.

Figure.3 Numbers of papillomas of anionic DPPG-paclitaxel nanoparticles (paclitaxel incorporated in "anionic bicelles")-treated mouse (●) and paclitaxel-treated mouse (control, □).



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 as chemo-preventive and anti-skin cancer agents, which can infiltrate into epidermis layer decreasing numbers of papillomas.

Nevertheless, many reports mentioned cleaning the samples objects with water before using them a second time in the test, but only a little reported using an extra copy of the sample object or cleaning the object with ethanol to remove the potential scent marks. In all reports the time of exploration was the basis of the test readout. The novelty preference index (NPI) of the mouse, which was administered memantine (memary) gluco-oligosaccharides orally, was 67%. On the other hand, the NPI of control mouse, that was administered memantine itself orally, was 55%. Although the NPI of the mouse injected memantine (memary) gluco-oligosaccharides intraperitoneally was 69%, the NPI of control mouse administered memantine itself intraperitoneally was 58%. Memantine (memary) gluco-oligosaccharides enhanced spatial learning of the mouse.

In vivo rotation test of memantine (memary) gluco-oligosaccharides

The rotation test is a standard measurement of 6-OHDA lesion efficacy. After unilateral median forebrain bundle DA depletion, a postural bias towards the side of the lesion is exhibited. Ipsilateral rotation is driven by an imbalance of DA between hemispheres generating decreased movement on the side of lesion. Spontaneous rotations ipsilateral to the 6-OHDA-lesioned side of 6-OHDA-induced hemi-parkinsonism mice were represented as a percent of the total rotations. The group of PBS infusion was tested. Although its rate of ipsilateral turns resulted in 89%, that of intra-peritoneally-memantine (memary) gluco-oligo-saccharides-treated 6-OHDA-induced hemi-parkinsonism mice decreased to 51%. The intraperitoneally injected memantine (memary) experiment showed 77%. The rate of ipsilateral turns of orally-memantine (memary) gluco-oligosaccharides-treated 6-OHDA-induced hemi-parkinsonism mice was 55%. The orally injected memantine (memary) experiment showed 80%. The finding obtained here demonstrated that memantine (memary) gluco-

oligosaccharides treated the Parkinson's disease (PD) improving the parkinsonian sign, i.e., drive of ipsilateral turns.

In vivo hind limb steps test of memantine (memary) gluco-oligosaccharides

Total number of steps were counted with both ipsi-and contra-lateral hind limbs. Spontaneous contralateral hind limb steps were indicated as % of the total steps. The contralateral hind limb steps in the PBS infusion test resulted in 27%. On the other hand, the contralateral hind limb steps of intraperitoneally-memantine (memary) gluco-oligosaccharides-treated 6-OHDA-induced hemi-parkinsonism mice increased to 52%. The intraperitoneally injected memantine (memary) control experiment showed 36%. The result of orally-memantine (memary) gluco-oligosaccharides-treated 6-OHDA-induced hemi-parkinsonism mice was 50%. The orally injected memantine (memary) experiment showed 33%. This result indicated that memantine (memary) gluco-oligosaccharides improved the movement of contralateral hind limb, showing that memantine (memary) gluco-oligosaccharides treated the Parkinson's disease (PD) improving the parkinsonian sign, i.e., inhibition of contralateral hind limb steps.

Anti-allergic activity of α-tocopherol glycoside and daidzein glycoside and glycitein glycoside against glutenin and globulin

The inhibitory activities of α-tocopherol, daidzein, glycitein α-tocopherol glucoside, daidzein glucoside, and glycitein glucoside for O_2^- generation from rat neutrophils were 33%, 29%, 30%, 58%, 50%, and 55% inhibition, respectively. Compound 48/80-induced histamine release from rat peritoneal mast cells was inhibited by α-tocopherol glucoside with a %inhibition of 83%. Daidzein glucoside (62% inhibition) and glycitein glucoside (66% inhibition) had strong inhibitory activity toward histamine release. The anti-allergic actions of the glycosides were caused by inhibition of histamine release with these compounds owing to their inhibitory aspects for O_2^- generation from rat neutrophils. The effects of α-tocopherol glucoside and α-tocopherol on immunoglobulin E (IgE) antibody formation were investigated by an *In vivo* bioassay using glutenin as an antigen. It was found that α-tocopherol glucoside showed stronger anti-allergic activity (IgE level 96) against glutenin than α-tocopherol (IgE level 192). When gliadin was used as the antigen, α-tocopherol glucoside showed stronger anti-allergic activity (IgE level 128) against gliadin than α-tocopherol (IgE level 192). On the other hand, IgE levels of daidzein glucoside and daidzein against 7S-globulin were 128 and 256. IgE levels of glycitein glucoside and glycitein against 7S-globulin were 128 and 192. Daidzein glucoside and glycitein glucoside showed higher anti-allergic activity against 7S-globulin than the aglycons, daidzein and glycitein, respectively; the suppression activity toward IgE formations of the glycosides was higher than that of the corresponding aglycons.

In conclusion, paclitaxel nanoparticles stabilized by anionic phospholipids of DPPG, “anionic bicelles” (particle size: 12 nm), were added to rat skin tissue, they penetrated the skin barrier of stratum corneum (intercellular space: ca. 100 nm). Applications of paclitaxel, which has no skin permeability, to anti-skin cancer materials have been still challenging because of its difficulty in transdermal delivery. 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.

Previous studies have reported that the glucosides of ketoprofen and indomethacin could significantly inhibit the glucose transporter (GluT1)-mediated uptake of glucose, indicating its affinity to the transporter (Gynther *et al.,* 2009; Berardi *et al.,* 2009). In addition, these glucoconjugates could temperature-dependently penetrate the BBB, indicating that the glucosylation of drugs enhances their BBB-crossing ability and that the brain uptake of the conjugates is carrier-mediated (Gynther *et al.,* 2009). Consistent with these studies, we also observed that memantine (memary) gluco-oligosaccharides can cross the BBB in the mouse brain and be incorporated into brain tissue. It has been shown anti-dementia drug such as memantine decreases β-amyloid levels via increase in secretion of amyloid precursor protein and activation of α-secretase (Niles *et al.,* 2006; Shan *et al.,* 2014; Hashemi *et al.,* 2022). On the other hand, the hippocampus is a critical brain area for cognitive and memory functions, making it a sensitive area in Alzheimer’s (Berardi *et al.,* 2009). Anti dementia drug

has been shown to improve learning and memory in several pharmacological models of Alzheimer's disease. For example, the study of the effects of such drug on locomotor activity, social behavior, and spatial learning assessed in a transgenic mouse model of Alzheimer's disease indicated that it improves hippocampus-based spatial learning in a transgenic mouse model of Alzheimer's disease without producing nonspecific effects on locomotion/exploratory activity (Evers *et al.,* 2004; Minkeviciene *et al.,* 2004; Rimando *et al.,* 2004). These previous findings are consistent with our study, which suggests that memantine (memary) gluco-oligosaccharides are chemopreventive agents that can protect neurons against the β-amyloid-induced disruption of spatial learning and memory in the hippocampus of SAMP8 and enhance spatial learning.

Therefore, based on these results, our findings suggest that the gluco-oligosaccharide modification of neuroprotective chemicals, such as curcumin, enhances their crossing ability through the BBB in the brain, thus, proposing that the brain–drug-delivery technique of neuroprotective chemicals by glycoside (gluco-oligosaccharide) modification is useful for preparing new anti-dementia drugs. In addition, this study indicates that memantine (memary) gluco-oligosaccharides can treat the Parkinson's disease (PD) improving the parkinsonian signs, i.e., drive of ipsilateral turns and inhibition of contralateral hind limb steps.

It is known that glutenin, gliadin, and 7S-globulin are allergic compounds included in wheat flour and soybean, respectively. α-Tocopherol is a component in wheat and daidzein and glycitein in soybean. α-Tocopherol glucoside, daidzein glucoside, and glycitein glucoside would be potent anti-allergic food additives. Our findings indicate that suppression of O_2^- generation caused inhibition of signal transduction of histamine release, resulting in reduction of IgE antibody formation.

Further studies on the anti-skin cancer property of anionic DPPG-paclitaxel nanoparticles, “anionic bicelles”, are now in progress in our laboratory.

Author Contributions

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. Yuya Kiriake: Validation, formal analysis, writing—reviewing. Daisuke Sato: Conceptualization, methodology, data curation, supervision, writing—reviewing the final version of the manuscript.

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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