JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 863–870

Received January 7, 2008
Accepted March 20, 2008
DOI 10.1211/jpp.60.7.0007
ISSN 0022-3573

Synthesis and anti-melanogenic effects of lipoic acid–polyethylene glycol ester

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Abstract

To develop a new potent anti-melanogenic agent, we have conjugated lipoic acid (LA) to poly (ethylene) glycol (PEG) of molecular weight 2000 and examined the effects on inhibition of tyrosinase activity and melanin synthesis in B16F10 melanoma cells. The water-soluble LA–PEG 2000 was synthesized from LA and methylated PEG by an esterification reaction in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Synthetic LA–PEG 2000 was confirmed by IR and ^{1}H-NMR spectroscopy. The new conjugate is a highly water-soluble molecule, which has lower cell cytotoxicity than LA. Treatment with LA–PEG 2000 significantly suppressed the biosynthesis of melanin by up to 63% at 0.25 mM and reduced tyrosinase activity by up to 80% at 0.50 mM in B16F10 melanoma cells. Furthermore, Western blot and RT-PCR studies indicated that treatment with LA–PEG 2000 decreased the level of tyrosinase, which is a melanogenic enzyme. Taken together, these results suggest that LA–PEG 2000 may inhibit melanin biosynthesis by down-regulating levels and expression of tyrosinase activity. Therefore, LA–PEG 2000 can be used effectively as a new agent to inhibit melanogenesis, with lower cytotoxicity than LA (parent molecule) in B16F10 melanoma cells.

Introduction

Primarily synthesized within melanocytes, melanin contributes to the pigmentation of the skin, hair, brain and eyes. The complex regulatory control of the biosynthetic machinery involved in melanogenesis includes receptor-mediated pathways activated by hormones, neurotransmitters, cytokines and growth factors, as well as receptor-independent mechanisms activated or modified by nutrients, micromolecules, microelements, pH, cation and anion concentrations, and the oxidoreductive potential (Slominski et al 2004). Melanin production plays an important role in prevention of sun-induced skin injury (Hill et al 1997). However, abnormal hyperpigmentation such as freckles, chloasma, lentigines and other forms of melanin hyperpigmentation can be serious aesthetic problems (Gilchrest 1996).

Melanin synthesis is mainly regulated by tyrosinase, which catalyses the rate-limiting reactions (tyrosine hydroxylation to L-3, 4-dihydroxyphenylalanine [L-DOPA], and oxidation of L-DOPA to DOPA-quinone) that are common to both eu- and pheomelanogenesis. The subsequent steps after the formation of DOPA-quinone are responsible for switching between these two types of melanin. The progression of these steps, including spontaneous chemical reactions, depends on the ratio of sulfhydryl compounds such as cysteine and/or glutathione (GSH) within melanocytes. In the absence of cysteine and/or GSH, DOPA-quinone is oxidized to form DOPA-chrome as the intermediate product of eumelanin, which results in the advance of eumelanogenesis. In the presence of these compounds, DOPA-quinone is coupled with their SH groups to form cysteinyl DOPA as a precursor of sulfur-containing pigment known as pheomelanin, which corresponds to the progress of pheomelanogenesis. Thus, in addition to tyrosinase, thiols that capture DOPA-quinone are thought to be essential for melanogenesis (Carter & Shuster 1978; Iozumi et al 1993; Kameyama et al 1993; Hearing 1999; Briganti et al 2003).

α-Lipoic acid (LA; thioctic acid), a dithiol compound derived from octanoic acid, is a necessary cofactor for mitochondrial enzymes and is also a bioavailable compound that is capable of scavenging a number of free radicals (Packer et al 1995; Packer 1998). LA is

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Acknowledgements: This study was supported by grants from the Ministry of Commerce, Industry and Energy (MOCIE) of Korea (70001410).

readily absorbed and converted to a reduced form, dihydrolipioic acid (DHLA), in various tissues. LA and DHLA are both antioxidants and directly scavenge reactive oxygen and nitrogen species (Kagan et al 1992; Scott et al 1994). LA regulates and synthesizes endogenous antioxidants (i.e. GSH and vitamin E) and has metal ion chelating activity (Scott et al 1994; Tsuji-Naito et al 2006). Because of its redox activity, LA also appears to stimulate certain signal transduction pathways and redox-sensitive gene expression. Furthermore, LA modulates melanin synthesis by decreasing levels of microphthalmia-associated transcription factors (MITF) and subsequently those of melanogenic enzymes (Lin et al 2002).

Having diverse biological functions, LA is being widely used in neurodegenerative diseases, HIV infection and as an anti-cancer drug. However, medical applications of LA are limited because it is difficult to solubilize in water (Segall et al 2004). To overcome this defect, synthetic and natural polymers such as the non-ionic water-soluble polymer poly (ethylene) glycol (PEG) are used as carriers for many drugs, attached via their end groups, (e.g. aspirin, penicillin V, quinidine) to solubilize molecules that are normally insoluble under physiological conditions (Zalipsky et al 1983; Paul et al 2005). In general, PEG is formed by a process of linking repeating units of ethylene glycol to form polymers with linear or branched shapes of different molecular masses. These PEG structures are then chemically attached to the drug of choice in a process called pegylation. Studies of PEG in solution reveal that each ethylene glycol subunit is tightly associated with two or three water molecules. There are several derivatives of LA conjugated to an antioxidant, such as Trolox®, a water-soluble analogue of vitamin E, to improve the effect of LA (Koufaki et al 2001). In a similar way, Harnett et al (2002) have synthesized a hybrid of lipoate and of a nitric oxide synthase inhibitor that is capable of protecting neuronal cells against glutamate toxicity.

In the present study, we describe the synthesis and characterization of an LA–PEG conjugate and confirm that the attachment of PEG to LA enhances the biological efficacy of LA. Here we present the effects of PEG conjugation to LA on its physical and biological properties. To the best of our knowledge, this is the first report describing the synthesis of an LA–PEG ester derivative and its anti-melanogenic effects.

Materials and Methods

Instrumentation and general techniques

^{1}H-NMR spectra were recorded on a Brucker 600 MHz spectrometer (Brucker, Karlsruhe, Germany), with $CDCl_3$ as an internal standard. IR absorption spectra were recorded on a JASCO-5300 (Jasco, Tokyo, Japan). The HPLC system consisted of a Waters 2695 system and an Xterra RP18 column (Waters, 5 μm, 2.1 × 50 mm) with a Waters 996 photodiode array detector or electro light-scattering detector (Alltech, USA). Column chromatography was performed using a column packed with Silicagel 60 (70-230 Mesh, Merck, Whitehouse Station, NJ, USA), and thin-layer chromatograms (TLC) were run on a TLC glass sheet coated with silica gel 60 F_{254} (Merck).

PEG, MW 2000 (n=43), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 4-dimethylaminopyridine (DMAP), LA, arbutin and porcine esterase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Solvents were laboratory grade or better. Antibodies against tyrosinase (sc-7833), tyrosinase-related protein (TRP)-1 (sc-10443), TRP-2 (sc-10452) and actin (sc-1616) were purchased from Santa Cruz Biotechnology (CA, USA).

Synthesis of LA–PEG ester derivative

To a solution of LA (1.00 g, 4.85 mM) in 15 mL dichloromethane (DCM) was added a solution of EDC (0.93 g, 4.85 mM) in 10 mL DCM and DMAP (0.016 g, 0.1 mM), and stirred for 20 min. A solution of PEG (8.81 g, 4.41 mM) in 10 mL DCM was added to the stirred mixture, and stirred for a further 24 h at room temperature. The residual solvent was removed at reduced pressure. The resulting solid was dissolved in 50 mL DCM and washed three times with the same volume of water to remove the side products. The reaction mixture was evaporated and dried in a freeze dryer. After dissolving the resulting solid in a small amount of DCM, diethyl ether was added to the resulting solution to precipitate lipoate ester at −10 to −20°C under vigorous stirring conditions. The mixture was filtered under reduced pressure to obtain a white solid with a slight yellow colour (99.9%, 7.1 g). IR ν_{max} (KBr) cm^{-1}: 2884, 1734, 1467, 1100. ^{1}H-NMR ($CDCl_3$) δ: 4.21–4.25 (2H, m, (-(O=C)-O-CH_2-CH_2-O)), 3.38–3.43 (3H, m, (CH_3-O-), 3.55–3.71 (182H, m, (-(CH_2-CH_2-O)n)), 3.05–3.22 (2H, m, (-CH_2-CH_2-S-S)), 2.32–2.52 (1H, m, (-CH_2-CH-S-S)), 2.33–2.38 (2H, m, (-CH_2-CH_2-(C=O)-)), 1.84–1.96 (1H, m, (-CH_2- CH_2-S-S-)), 1.60–1.74 (5H, m, (-CH_2-CH_2-(C=O)-), (-CH_2-CH_2-(C=O)-), (-S-CH-(CH_2-CH_2-S-)-CH_2-)).

Measurement of enzymatic hydrolysis and solubility

The commercial esterase was dissolved in isotonic buffer (pH 8.0) to give a concentration of 0.1 mg mL^{-1}. A stock solution of LA–PEG 2000 was prepared by dissolving the appropriate amount of prodrug in distilled water in order to obtain a concentration of 20 mM and kept in screw-capped vials at 4°C. The reaction was initiated by adding 20 μL LA–PEG 2000 stock solution to 1 mL esterase solution in a screw-capped vial. The solutions were kept in a water bath at 37°C. After filtration, samples were analysed immediately by HPLC (Johansen et al 1986; Wong et al 1989). The water solubility of LA–PEG and LA was tested by HPLC analysis after filtering the saturated water solution at 25°C.

Cell culture

A B16F10 mouse melanoma cell line procured from the Korean Cell Line Bank was used in this study. Cells were grown in DMEM supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 1% penicillin–streptomycin (Gibco BRL, NY, USA) and 200 nM α-melanocyte stimulating hormone (α-MSH, Sigma) at 37°C in a humidified incubator with 5% CO_2.

Measurement of cell viability

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was performed as described by Mosmann (1983). Briefly, 1×10^5 cells per well were plated into 24-well plates. After the required incubation with LA–PEG ester for 24 h, MTT solutions ($5\ \mu g\,mL^{-1}$) were added, and the insoluble derivative formed by the cellular dehydrogenase enzyme was solubilized with acid-isopropanol (0.04 M HCl in isopropanol), and absorbance was measured at 565 nm using a microplate reader.

Measurement of melanin content

Melanin content was estimated using a modification of the method reported by Bilodeau et al (2001). B16F10 melanoma cells were seeded into six-well plates at a density of 3×10^5 cells per well and cultured. After 24 h, the cells were washed and placed in culture medium with different concentrations of test compounds for 72 h. Cells were then collected and counted, washed with phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 5 min to collect the cell pellet, which was dissolved in 1 mL homogenization buffer (50 mM sodium phosphate pH 6.5, 1% Triton X-100, 2 mM phenylmethanesulfonyl fluoride (PMSF)), and 200 μL 1 M NaOH (+ 10% DMSO). The absorbance at 405 nm was measured and the melanin concentration calculated from a standard curve created using synthetic melanin (Sigma).

Measurement of tyrosinase activity

Tyrosinase activity was measured according to the method of Kim et al (2005) with slight modifications. Tyrosinase activity, using L-DOPA as the substrate, was assayed spectrophotometrically. The assay medium, consisting of 0.1 mL mushroom tyrosinase solution ($625\ U\ mL^{-1}$), 0.9 mL 1/15 M PBS buffer solution (2.0 mM) and 1.0 mL of sample solution containing 5 mM DMSO, was mixed and pre-incubated at 25°C for 10 min. Then, a reaction was carried out by adding 0.03% L-DOPA solution. A control reaction (A) was conducted without the test sample, and a blank reaction (B) was used for non-active heated mushroom tyrosinase. The absorbance was measured at 475 nm after incubation, giving the sample value as C. The percentage of inhibition of tyrosinase was calculated as follows: tyrosinase inhibition $(\%) = [(A–C)/(A–B)] \times 100$, where the absorbance value for 5 min incubation was determined. Arbutin was used as the positive control.

Cellular tyrosinase activity was measured using a modification of the methods reported by Pawelk (1979) and Pomerantz (1963). B16F10 melanoma cells were seeded into six-well plates at a density of 5×10^5 cells per well and cultured for 24 h. Following treatment with various concentrations of samples for 24 h, cells were collected and resuspended. After incubation with 0.1 M sodium phosphate buffer, pH 6.8, containing 0.2% L-DOPA at 37°C for 2 h, the amount of DOPA-chrome in the reaction mixture was determined by measuring the absorbance at 490 nm.

Western blot analysis

B16F10 melanoma cells were seeded into six-well plates at a density of 3×10^5 cells per well and cultured for 24 h. Cells were then treated with various concentrations of samples for 48 h and then harvested. The cells were treated with radioimmunoprecipitation assay buffer containing 10 mM sodium fluoride, 0.1% SDS, 1% NP-40, 1 mM DTT, 500 μM sodium orthovanadate, $10\ \mu g\ mL^{-1}$ aprotinin, $10\ \mu g\ mL^{-1}$ leupeptin and 1 mM PMSF. The solubilized proteins were separated on 12% SDS–polyacrylamide gels and transferred to PVDF membrane and incubated overnight with 3% powdered milk in Tris-buffered salt (TBS) containing 0.1% Tween-20 at 4°C. The membrane was rinsed in four changes of TBS with Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), incubated twice for 5 min and twice for 10 min in fresh washing buffer. It was then incubated for 2 h with blocking solution containing 1:500 dilution of primary antibody (tyrosinase, TRP-1, TRP-2, actin; Santa Cruz Biotechnology). After four washes, the membrane was incubated for 1 h in horseradish peroxidase-conjugated anti-goat IgG secondary antibody (1:2000, Santa Cruz Biotechnology) and developed using enhanced chemiluminescence (ECL Western blot analysis system kit, Amersham Biosciences, Little Chalfont, UK). The Western blot was analysed by scanning with a UMAX PowerLook 1120 and digitalizing using image analysis software (Kodak 1D, Eastman Kodak Co., NY, USA).

RT-PCR analysis

B16F10 melanoma cells were seeded into six-well plates at a density of 3×10^5 cells per well and cultured for 24 h. Cells were harvested after treatment with various concentrations of samples for 24 h. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, MD, USA) from the B16F10 melanoma cells. Then, 1 μg of RNA was reverse transcribed using the Omniscript reverse transcription system (Qiagen, Hilden, Germany). The cDNA obtained was amplified with the following primers: tyrosinase (276 bp product) forward 5′-GAGAAGCGAGTCTTGATTAG-3′ and reverse 5′-TGGTGCTTCATGGGCAAAATC-3′; TRP-1 (268 bp product) forward 5′-GCTGCAGGAGCCTTCTTTCTC-3′ and reverse 5′-AAGACGCTGCACTGCTGGTCT-3′; TRP-2 (218 bp product) forward 5′-CCTGTCTCTCCAGAAGTTTG-3′ and reverse 5′-CGTCTGTAAAAGAGTGGAGG-3′; GAPDH (546 bp product) forward 5′-ATTGTTGCCATCAATGACCC-3′ and reverse 5′-AGTAGAGGCAGGGATGATGT-3′. The PCR conditions were 25 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. The resulting PCR products were visualized by electrophoretic separation on 1.5% agarose gel with ethidium bromide staining. Specific primers for GAPDH were used as a control.

Statistical analysis

All data from in-vitro experiments are presented as mean ± s.d. One-way analysis of variance and Duncan's test were used for multiple comparisons (SPSS program, ver 10.0). *P* values below 0.05 were regarded as significant.

Results and Discussion

Synthesis of LA–PEG ester derivative

The general strategy of the synthesis is shown in Figure 1. For the synthesis of LA–PEG ester, linear methoxy PEG 2000 was used, which has one end functional group, to suppress the synthesis of the byproduct. Purification problems were avoided by separation of unreacted PEG from LA–PEG 2000, which has similar physicochemical properties. The attachment of PEG to the carboxyl group of LA was performed by means of EDC and DMAP. DMAP is widely used as a hypernucleophilic acylation catalyst. EDC catalyses the formation of amide bonds between carboxylic acids and amines by activating the carboxyl group to form a urea derivative. This can be used to make ester links from acid and alcohols. The reaction was ended with a good yield (90%) when the PEG spot was not detected on the TLC plate. The products were washed with water and purified from the residual LA and other reactants by recrystallization from ether/methylene chloride and reverse-phase chromatography (yield 82.3%). The absence of reactive molecules was verified by HPLC. Since PEG could not be detected by UV spectroscopy, we used an electro light-scattering detector for PEG, and a photodiode array detector for LA (data not shown). We found easy, high-yield synthesis and good water solubility of LA–PEG (Table 1).

The role of various water-soluble carriers was studied for dissolution enhancement of a poorly soluble model drug. PEG has been found to facilitate the solubilization of water-insoluble materials in physiological medium (Yang et al 2004). PEG has been used to help disperse lipophilic materials such as LA.

The esterification of the end group of PEG was confirmed by FT-IR spectroscopy and ^{1}H-NMR. The ^{1}H-NMR adduct spectrum indicated the presence of methylene protons ascribed to the PEG moiety ($\delta = 3.55$–3.71) and LA moiety ($\delta = 1.60$–3.55). Figure 1 shows the FT-IR spectra of LA (Figure 2a), mPEG 2000 (Figure 2b), and LA–PEG 2000 (Figure 2c). The spectra demonstrate the introduction of the acetyl group, as indicated by esterification of the end group in the PEG and was confirmed at an absorption of 1734 cm^{-1} (C = O of COOH). The signals typically corresponded to the values reported in the literature (He et al 2005).

Enzymatic hydrolysis of LA–PEG ester

A molecule with high molecular weight would not be expected to penetrate the intact skin barrier and reach the target cells. In the skin, there are a lot of enzymes (esterases etc.). A prerequisite for success in the use of prodrug is that reconversion of the prodrug into the parent molecule occurs in the skin. In our experiments we assessed the enzymatic cleavage of ester using porcine liver esterases, which are regarded as a good model for skin enzymatic activity (Johansen et al 1986; Wong et al 1989). As shown in Table 1, the synthesized ester (LA–PEG) had a notable stability in phosphate buffer at pH 6.0 and was readily hydrolysed by porcine esterase. Skin esterases produce free LA, which could penetrate to deeper skin layers.

Cytotoxicity of LA–PEG compared with LA

To investigate cell viability, B16F10 melanoma cells were cultured with LA–PEG 2000 and LA at concentrations ranging from 0.1 to 5.0 mM for 24 h. When the cells were exposed to various concentration of LA (0.1–5 mM), the cell viability decreased gradually. In contrast, when LA–PEG 2000 (0.1–5 mM) was added to the culture medium of B16F10 melanoma cells, cell viabilities were not significantly different from the

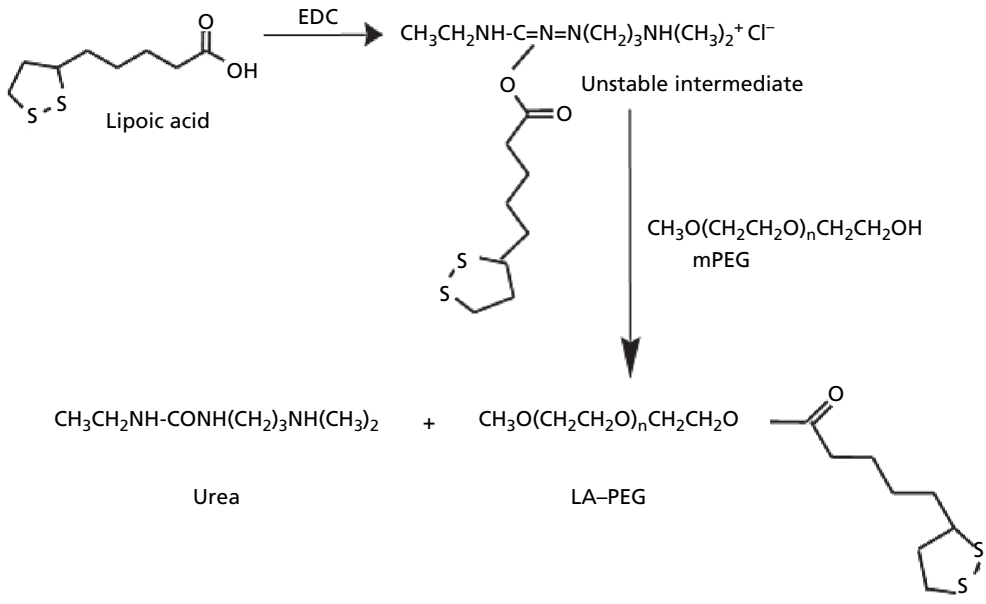

Figure 1 Synthesis of lipoic acid–polyethylene glycol (LA–PEG) conjugate. EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

Table 1 Molecular weight (MW), synthetic yield, water solubility at 25°C and the half-life $t_{1/2}$ of the enzymatic hydrolysis of LA–PEG ester

Compound	MW	Synthetic yield (%)	Solubility ($mg\,mL^{-1}$)	$t_{1/2}$ (h)	
				Buffer, pH 8.0	Porcine esterase
LA	206	–	0	–	–
LA–PEG 2000	2000	82.3	350	1440	3.5

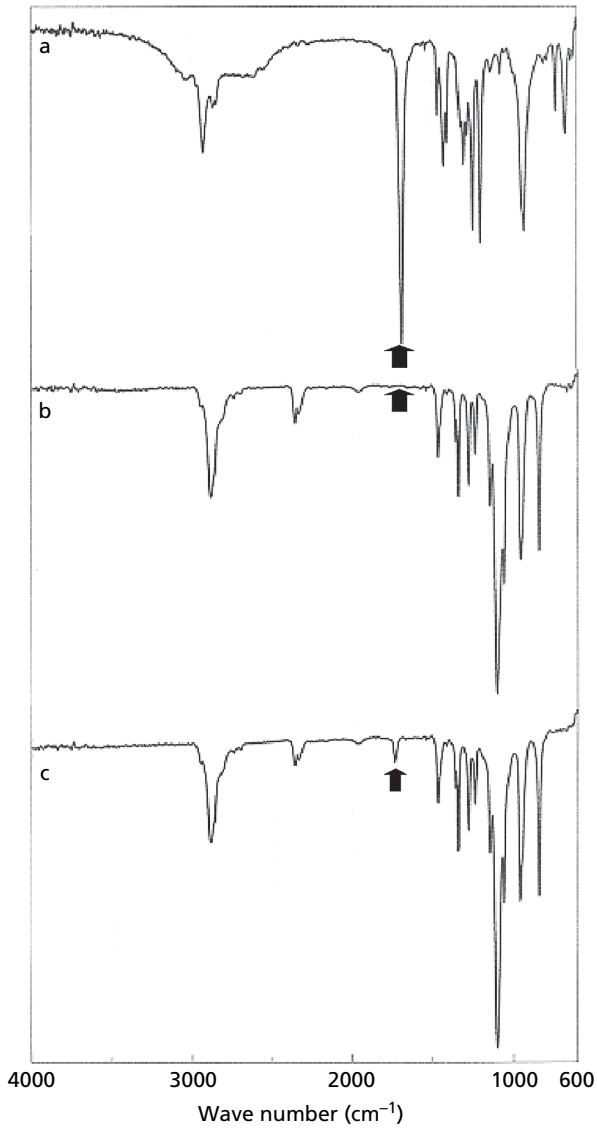

Figure 2 FT-IR spectra of (a) lipoic acid (LA), (b) mPEG 2000 and (c) LA–PEG 2000. The spectra demonstrate the introduction of the acetyl group, as indicated by esterification of the end group in the PEG; the arrow shows that it was confirmed at an absorption of 1734 cm^{-1} (C=O of COOH).

controls (Figure 2). From our results we conclude that LA–PEG 2000 has reduced the cell cytotoxicity of LA.

It has been reported that PEG can enhance the membrane resealing process to restore membrane integrity, and the effects of pegylation include decreased toxicity (Harris & Chess 2003). The PEG polymer, along with the associated water molecules, acts like a shield to protect the attached drug from enzyme degradation, rapid renal clearance and interactions with cell surface proteins, thereby limiting adverse immunological effects. Pegylated drugs are also more stable over a range of pH and temperature changes compared with their unpegylated counterparts (Harris & Chess 2003). We synthesized the LA–PEG by conjugating the carboxyl group of LA with PEG; the shielding of the acidic charge on LA could decrease the cell cytotoxicity.

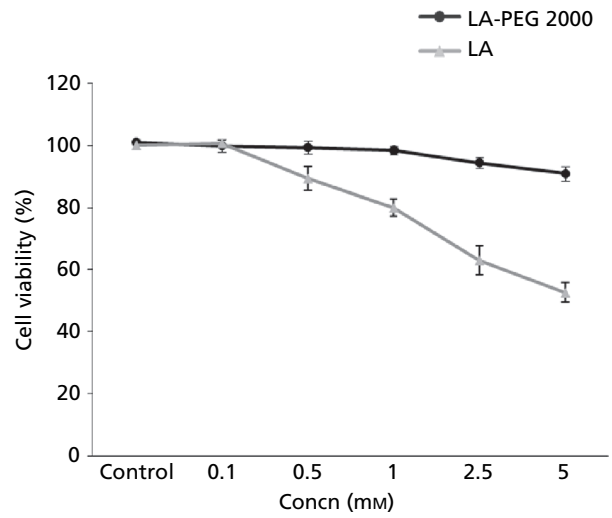

Figure 3 Effect of LA–PEG 2000 on relative cell viability in B16F10 melanoma cells. Cells were treated with various concentrations of LA–PEG 2000 for 24 h. The results are the mean ± s.d. of triplicate samples.

Effects of LA–PEG on melanin synthesis

We quantitatively examined the effect of LA–PEG 2000 on melanogenesis and compared the results with LA and α-arbutin. To assess the inhibitory activity of LA–PEG 2000 against melanin synthesis, we measured the level of melanin in B16F10 melanoma cells cultured with LA–PEG 2000 for 72 h. Melanin production was decreased to 20%, 62.8% and 71.4% at 0.1, 0.25 and 0.5 mM LA–PEG 2000, respectively. LA decreased the melanin content to 8.4%, 21.5% and 60.7% at 0.1, 0.25 and 0.5 mM, respectively (Figure 4). This result indicates that LA–PEG 2000 suppresses the synthesis of melanins more effectively than LA.

Harris & Chess (2003) reported that PEG is a biocompatible, non-toxic, non-immunogenic and water-soluble polymer with many uses in biomaterials. Synthesis of its derivatives is very important and is the first step in the application of PEG. When PEG is properly linked to LA, it modifies many of its features while the main biological functions may be maintained and improved. Pegylation increases the duration of LA release (Greenwald 2001; Harris & Chess 2003). The cell culture medium contained a lot of esterase from the cultured cells. Commercial esterase rapidly hydrolysed LA–PEG (Table 1). We couldn't compare the rate of cellular uptake of LA–PEG against LA because LA hydrolysed by commercial esterase was not detected, but we could analyse LA and

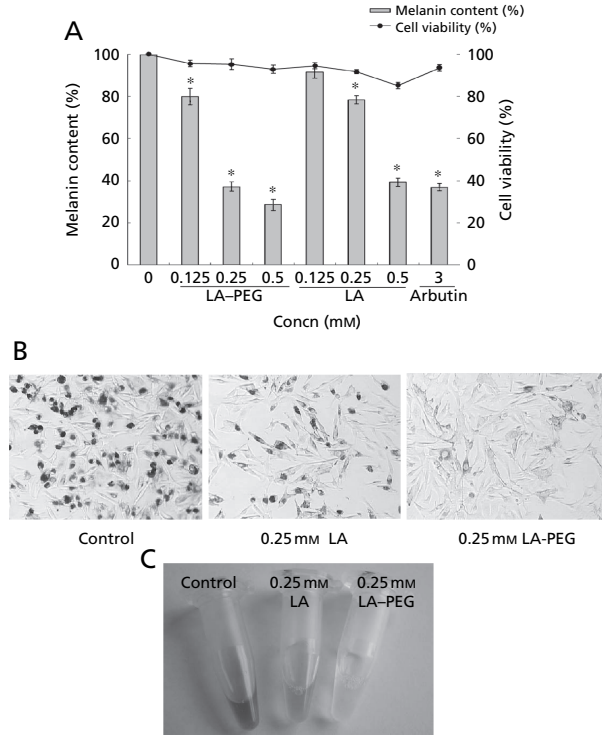

Figure 4 Effect of LA–PEG 2000 on melanin production in B16F10 melanoma cells. (a) Cells were treated with various concentrations of LA–PEG 2000 and LA for 72 h. Results are the mean ± s.d. of triplicate samples. $*P < 0.05$ compared with control. (B) Phase-contrast micrographs of B16F10 melanoma cells after treatment without or with LA and LA–PEG (0.25 mM). (C) Cells were treated with 0.25 mM LA and LA–PEG. The cells were suspended and then photographed.

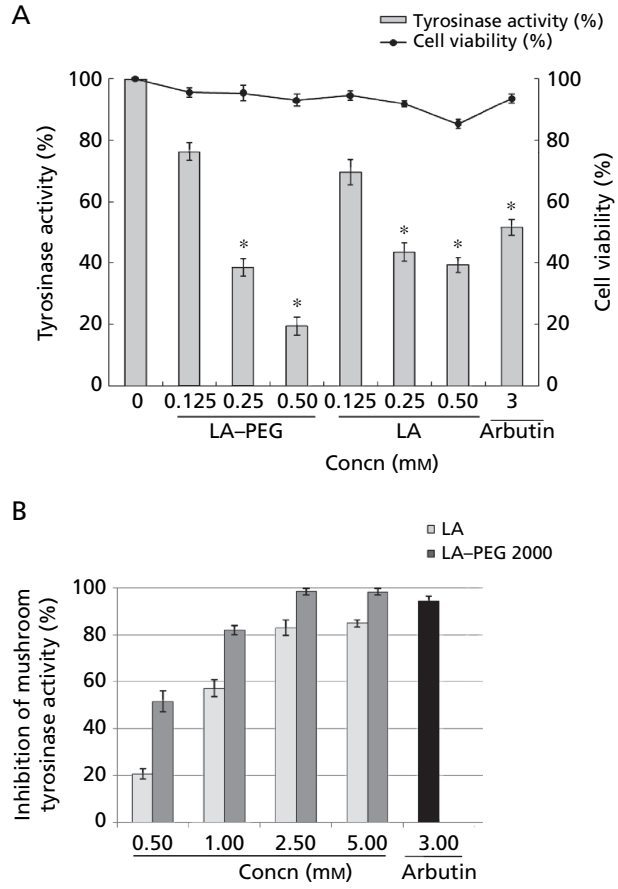

Figure 5 (a) Effect of LA–PEG 2000 on tyrosinase activity in B16F10 melanoma cells. The cells were incubated with LA–PEG 2000 for 24 h. Data are mean ± s.d. of triplicate samples. $*P < 0.05$ vs control. (b) Inhibitory effect of LA–PEG 2000 on mushroom tyrosinase activity.

LA–PEG by HPLC. However, we believe that LA–PEG is easily taken up into cultured cells after esterification by the esterases existing in the cell. The role of various water-soluble carriers was studied for dissolution enhancement of a poorly soluble model drug. PEG has been found to facilitate the solubilization of water-insoluble materials in physiological media (Yang et al 2004). PEG has been used to help disperse lipophilic materials such as LA.

In conclusion, after application on the skin, LA–PEG can be supplied more constantly in human cells with a higher anti-melanogenic activity compared with LA, because LA–PEG needs to be continuously hydrolysed into LA and PEG by esterase in the human cells and then absorbed in the cells, providing a slow and lasting release.

Effects of LA–PEG on tyrosinase activity

We examined the inhibitory effect of LA–PEG 2000 on the activity of tyrosinase in B16F10 melanoma cells and compared it with that of LA and arbutin. Tyrosinase activity was strongly influenced by LA–PEG 2000; LA–PEG 2000 had a stronger inhibitory effect on tyrosinase than arbutin. LA–PEG 2000 reduced tyrosinase activity up to 23.7%, 61.5% and 80.5% at 0.125, 0.25 and 0.5 mM, respectively, in B16F10 melanoma cells (Figure 5). LA inhibited tyrosinase activity by 30.4%, 56.4% and 60.6% at 0.125, 0.25 and 0.5 mM, respectively. Arbutin (3 mM) inhibited tyrosinase activity by 48.3%.

We tested the activity of the two compounds on purified tyrosinase (Figure 5B). LA and LA–PEG had consistent inhibitory effects on the purified commercial tyrosinase at concentrations of 1–5 mM.

Tyrosinase is the key enzyme for melanin biosynthesis, playing a role in oxidation from tyrosine to L-DOPA, and DOPA to DOPA-quinone. This process is a determinant of skin colour, and is also involved in local hyperpigmentation such as melasma, ephelis and lentigo. From our results, we supposed that LA–PEG 2000 inhibits melanin biosynthesis by regulating tyrosinase activity, with low cytotoxicity. Tsuji-Naito et al (2006) suggested that LA could modulate melanin synthesis because the sulfhydryl groups of the lipoyl motif react with DOPA-quinone to form lipoyl–DOPA conjugates. Bertazzo et al (2001) reported that, in the presence of PEG, the kinetics of tyrosinase-catalysed oligomerization of DOPA are slower than those observed in the absence of the polymer and suggested that the presence of PEG may affect the process of melanogenesis at molecular and intermolecular levels. But,

in our experiment, when PEG 2000 (0.1–0.5 mM) was added to the culture medium of B16 melanoma cells, tyrosinase activities were not significantly different from untreated controls (data not shown). After pegylation the LA–PEG 2000 may have a longer duration of activity resulting from slow release. Therefore, our results are assumed to be due to the synergistic effect of the conjugation of water-soluble PEG and LA.

Effects of LA–PEG on expression of tyrosinase, TRP-1 and TRP-2

To discover the effects of LA–PEG 2000 on tyrosinase and other melanogenic enzymes such as a TRP-1 and TRP-2, Western blot analysis was performed on these proteins. As shown in Figure 6, levels of tyrosinase and TRP-1 proteins were reduced by LA–PEG 2000, but TRP-2 was not affected by LA–PEG 2000. Lin et al (2002) found that LA and DHLA inhibit the expression of tyrosinase protein and reduce MITF promoter activity and MITF mRNA in B16 melanoma cells. In our experimental results, it is clear that the highly reduced melanin content and tyrosinase activity resulting from treatment with LA–PEG were due to reduced tyrosinase protein levels in B16F10 melanoma cells.

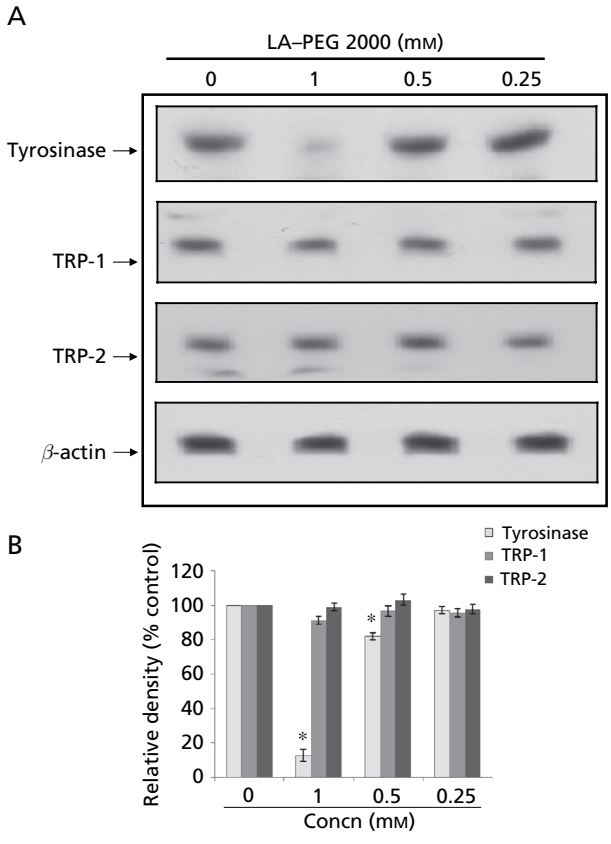

Figure 6 Effects of LA–PEG 2000 on levels of tyrosinase, TRP-1 and TRP-2 protein in B16F10 melanoma cells. (a) B16F10 melanoma cells were treated with various concentrations of LA–PEG 2000 for 48 h. (b) The relative intensity of the tyrosinase, TRP-1 and TRP-2 protein bands is expressed as a percentage of the signal intensity relative to untreated control. Each bar represents mean ± s.d. from triplicate experiments. $*P < 0.05$ vs controls.

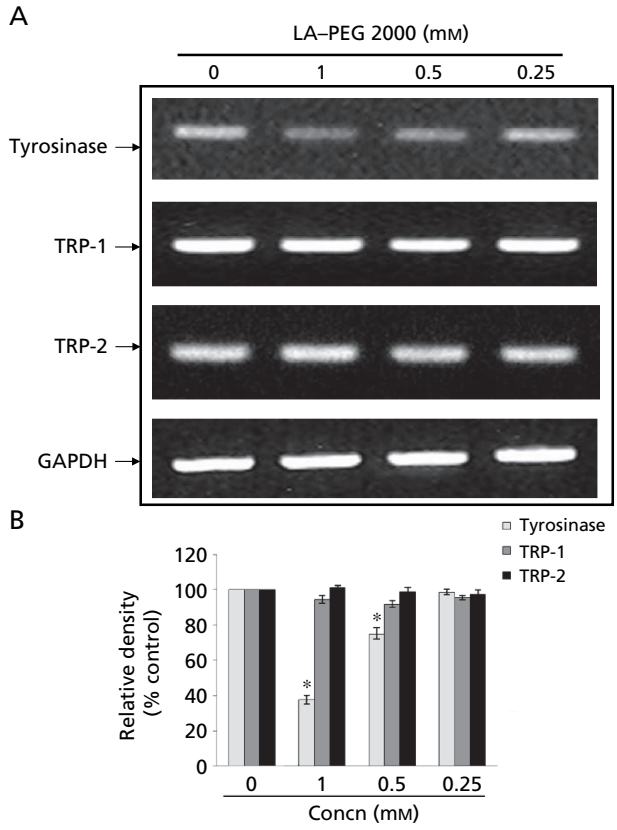

Figure 7 Effects of LA–PEG 2000 on tyrosinase, TRP-1, and TRP-2 mRNA levels in B16F10 melanoma cells. (a) B16F10 melanoma cells were treated with various concentrations of LA–PEG 2000 for 24 h. Total RNA extracted from B16F10 melanoma was analysed by RT-PCR. Similar results were observed in three independent experiments. (B) The relative intensity of the tyrosinase, TRP-1 and TRP-2 mRNA band, expressed as a percentage of the signal intensity relative to untreated controls. Each bar represents the mean ± s.d. from triplicate experiments. $*P < 0.05$ vs control.

To examine the effects of LA–PEG 2000 at the transcription level, RT-PCR analysis was done. Figure 7 shows the effects of LA–PEG 2000 on the expression of tyrosinase, TRP-1 and TRP-2 mRNA in B16F10 melanoma cells. With RT-PCR analysis, we confirmed that tyrosinase was controlled by LA–PEG 2000. These results suggest that the inhibitory effect of LA–PEG 2000 on melanin biosynthesis is related to the tyrosinase transcriptional level. LA–PEG 2000 may control the levels of tyrosinase protein and mRNA.

Conclusions

To evaluate LA–PEG 2000, a synthetic compound, for its potency in inhibiting hyperpigmentation, we compared its activities and cytotoxicity with LA, the original compound, and arbutin, a known depigmenting agent. The modified molecule was significantly less toxic than the original compound. Moreover, chemical modification led to enhanced water solubility.

LA–PEG 2000 retained a comparable level of biological activities to that of native LA. LA–PEG 2000 treatment significantly suppressed the biosynthesis of melanin by up to 63% at 0.25 mM in B16F10 melanoma cells, compared with 22% for LA at the same concentration. LA–PEG 2000 reduced tyrosinase activity up to 80% at 0.5 mM, compared with 61.5% for LA at the same concentration. These results suggest that LA–PEG 2000 is a more powerful anti-melanogenic agent than LA, and may inhibit melanin biosynthesis by down-regulating tyrosinase activity. In addition, tyrosinase protein and mRNA levels were decreased by LA–PEG 2000 treatment. These results suggested that the LA–PEG is an inhibitor of both tyrosinase activity and expression. Numerous pharmacological and cosmeceutic agents have been reported to inhibit tyrosinase and other melanogenic pathway targets, leading to skin lightening. We need to do more studies in this field.

In conclusion, LA conjugated with PEG has diverse applications as a new water-soluble anti-melanogenic agent, having potent inhibitory effects against tyrosinase activity and levels, with low cell cytotoxicity.

References

Bertazzo, A., Vogliardi, S., Favretto, D., Costa, C. V., Allegri, G., Traldi, P. (2001) Melanogenesis by tyrosinase action on 3,4-dihydroxyphenylalanine (DOPA) in the presence of polyethylene glycol: a matrix-assisted laser desorption/ionization mass spectrometric investigation. *Rapid Commun. Mass Spectrom*. **15**: 1061–1067

Bilodeau, M. L., Greulich, J. D., Hullinger, R. L., Bertolotto, C., Ballotti, R., Andrisani, O. M. (2001) BMP-2 stimulates *tyrosinase* gene expression and melanogenesis in differentiated melanocytes. *Pigmen. Cell Res*. **14**: 328–336

Briganti, S., Camera, E., Picardo, M. (2003) Chemical and instrumental approaches to treat hyperpigmentation. *Pigment Cell Res*. **16**: 101–110

Carter, R. J., Shuster, S. (1978) Melanocyte-stimulating hormone – mimetic action of the phenothiazines. *J. Pharm. Pharmacol*. **30**: 233–235

Gilchrest, B. A. (1996) A review of skin ageing and its medical therapy. *Br. J. Dermatol*. **135**: 867–875

Greenwald, R. B. (2001) PEG drugs: an overview. *J. Control. Release*. **74**: 159–171

Harnett, J. J., Auguet, M., Viossat, I., Dolo, C., Bigg, D., Chabrier, P. E. (2002) Novel lipoic acid analogues that inhibit nitric oxide synthase. *Bioorg. Med. Chem. Lett*. **12**: 1439–1442

Harris, J. M., Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discov*. **2**: 214–221

Hearing, V. J. (1999) Biochemical control of melanogenesis and melanosomal organization. *J. Invest. Dermatol. Symp. Proc*. **4**: 24–28

He, L., Robertson, J. W., Li, J., Karcher I., Schiller, S. M., Knoll, W., Naumann, R. (2005) Tethered bilayer lipid membranes based on monolayers of thiolipids mixed with a complementary dilution molecule. 1. Incorporation of channel peptides. *Langmuir* **21**: 11666–11672

Hill, H. Z., Li, W., Xin, P., Michell, D. L. (1997) Melanin: a two edged sword? *Pigment Cell Res*. **10**: 158–161

Iozumi, K., Hoganson, G. E., Pennella, R., Everett, M. A., Fuller, B. B. (1993) Role of tyrosinase as the determinant of pigmentation in cultured human melanocytes. *J. Invest. Dermatol*. **100**: 806–811

Johansen, M., Mollgaard, B., Wotton, P. K., Larsen, C. Hoelgaard, A. (1986) *In vitro* evaluation of dermal prodrug delivery – transport and bioconversion of a series of aliphatic esters of metronidazole, *Int. J. Pharm*. **32**: 199–206

Kagan, V. E., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell, R., Packer, L. (1992) Dihydrolipoic acid – a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxyl, ascorbyl and chromanoxyl radicals. *Biochem. Pharmacol*. **44**: 1637–1649

Kameyama, K., Takemura, T., Hamada, Y., Sakai, C., Kondoh, S., Nishiyama, S., Urabe, K., Hearing, V. J. (1993) Pigment production in murine melanoma cells is regulated by tyrosinase, tyrosinase-related protein 1 (TRP1), DOPA chrome tautomerase (TRP2), and a melanogenic inhibitor. *J. Invest. Dermatol*. **100**: 126–131

Kim, Y. J., No, J. K., Lee, J. H., Chung, H. Y. (2005) 4,4′-Dihydroxybiphenyl as a new potent tyrosinase inhibitor. *Biol. Pharm. Bull*. **28**: 323–327

Koufaki, M., Calogeropoulou, T., Detsi, A., Roditis, A., Kourounakis, A. P., Papazafiri, P., Tsiakitzis, K., Gaitanaki, C., Beis, I., Kourounakis, P. N. (2001) Novel potent inhibitors of lipid peroxidation with protective effects against reperfusion arrhythmias. *J. Med. Chem*. **44**: 4300–4303

Lin, C. B., Babiarz, L., Liebel, F., Roydon Price, E., Kizoulis, M., Gendimenico, G. J., Fisher, D. E., Seiberg, M. (2002) Modulation of microphthalmia-associated transcription factor gene expression alters skin pigmentation. *J. Invest. Dermatol*. **119**: 1330–1340

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immun. Methods* **65**: 55–63

Packer, L. (1998) alpha-Lipoic acid: a metabolic antioxidant which regulates NF-kappa B signal transduction and protects against oxidative injury. *Drug Metab. Rev*. **30**: 245–275

Packer, L., Witt, E. H., Tritschler, H. J. (1995) Alpha-lipoic acid as a biological antioxidant. *Free Radic. Biol. Med*. **19**: 227–250

Paul, A., Griffiths, P. C., James, R., Willock, D. J., Rogueda, P. G. (2005) Explaining the phase behaviour of the pharmaceutically relevant polymers poly(ethylene glycol) and poly(vinyl pyrrolidone) in semi-fluorinated liquids. *J. Pharm. Pharmacol*. **57**: 973–980

Pawelk, J. (1979) Melanoma cells in culture. *Methods Enzymol*. **58**: 564–570

Pomerantz, S. H. (1963) Separation, purification and properties of two tyrosinases from hamster melanoma. *J. Biol. Chem*. **238**: 2351–2357

Scott, B. C., Arouma, O. I., Evans, P. J., O'Neill, C., Van der Vliet, A., Cross, C. E., Tritschler, H., Halliwell, B. (1994) Lipoic and dihydrolipoic acids as antioxidants. A critical evaluation. *Free Radic. Res*. **20**: 119–133

Segall, A., Sosa, M., Alami, A., Enero, C., Hormaechea, F., Pizzorno, M. T., Bregni, C., Serrao, R. (2004) Stability study of lipoic acid in the presence of vitamins A and E in o/w emulsions for cosmetic application. *J. Cosmet. Sci*. **55**: 449–462

Slominski, A., Tobin, D. J., Shibahara, S., Wortsman, J. (2004) Melanin pigmentation in mammalian skin and its hormonal regulation, *Physiol. Rev*. **84**: 1155–1228

Tsuji-Naito, K., Hatani, T., Okada, T., Tehara, T. (2006) Evidence for covalent lipoyl adduction with dopaquinone following tyrosinase-catalyzed oxidation. *Biochem. Biophys. Res. Commun*. **343**: 15–20

Wong, O., Tsuzuki, N., Nghiem, B., Kuehnhoff, J., Itoh, T., Masaki, K., Huntington, J., Konishi, R., Rytting, J. H., Higuchi, T. (1989) Unsaturated cyclic ureas as new non-toxic biodegradable transdermal penetration enhancers. II. Evaluation study. *Int. J. Pharm*. **52**: 191–202

Yang, H., Morris, J. J., Lopina, S. T. (2004) Polyethylene glycol-polyamidoamine dendritic micelle as solubility enhancer and the effect of the length of polyethylene glycol arms on the solubility of pyrene in water, *J Colloid Interface Sci*. **273**: 148–154

Zalipsky, S., Gilon, C., Zilkha, A. (1983) Attachment of drugs to polyethylene glycols. *Eur. Polym. J*. **19**: 1177–1183