Inhibitory Effects of a Novel Ascorbic Derivative, Disodium Isostearyl 2-O-1-A scorbyl Phosphate on Melanogenesis

Sanae Matsuda, Hiroharu Shimayama, Masayoshi Hisama, Mamitaro Ohtsuki, and Masahiro Iwaki

a Central Research Center, Toyo Beauty Co., Ltd.; 3–13–8 Higashinakamoto, Higashinari-ku, Osaka 537–0021, Japan; b Department of Dermatology, Jichi Medical University; 3311–1 Yakushiji, Shimoisuke, Tochigi 329–0498, Japan; and c Faculty of Pharmaceutical Sciences, Kinki University; 3–4–1 Kowakae, Higashi-Osaka, Osaka 577–8502, Japan.

Received October 22, 2007; accepted December 28, 2007

We investigated the inhibitory effects of a novel amphiphilic ascorbic derivative, disodium isostearyl 2-O-1-ascorbyl phosphate (VCP-IS-2Na), synthesized from a hydrophobic ascorbic derivative, sodium-2-O-1-ascorbyl phosphate (VCP-Na), on melanogenesis in cultured human melanoma cells, normal human melanocytes, and three-dimensional cultured human skin models. Melanin synthesis in melanoma cells treated with VCP-IS-2Na at 300 μM and melanocytes treated with VCP-IS-2Na at 100 μM decreased to 23% and 52% of that in non-treated cells, respectively, and the cell viability was not affected. VCP-IS-2Na also significantly suppressed the cellular tyrosinase activity of melanoma cells and melanocytes. Melanin synthesis in human skin models was evaluated by macro- and microscopic observations of its pigmentation and quantitative measurements of melanin. Treatment of the human skin models with 1.0% VCP-IS-2Na did not inhibit cell viability, while melanin synthesis was decreased to 21% of that in the control. In contrast, l-ascorbic acid (VC) and VCP-Na did not seem to inhibit melanin synthesis and cellular tyrosinase activity. These results indicate that VCP-IS-2Na may be an effective whitening agent for the skin, and we expect the application of VCP-IS-2Na in the cosmetic industry.

Key words disodium isostearyl 2-O-1-ascorbyl phosphate; amphiphilic ascorbate; melanogenesis; human tyrosinase; three-dimensional human skin model

Skin color is mainly determined by the amount of melanin present in the surface of the skin. Melanin is synthesized in melanocytes, which are normally found in the epidermal basal layer. Within melanocytes, melanin is bound to a protein matrix to form melanosomes. In the melanosomes, tyrosinase converts tyrosine to eumelanin or pheomelanin through the pathways of melanin biosynthesis. By blocking the pathways at various points, skin depigmentation agents can inhibit melanin biosynthesis, and can be used to treat local hyperpigmentation or spots, which are caused by a local increase in melanin synthesis or uneven distribution. In the last decade, a number of depigmentation agents have been developed and utilized in cosmetics and pharmaceuticals for external use.

L-Ascorbic acid (VC) has various physiological and pharmacological functions in collagen synthesis, anti-oxidation, intestinal absorption of iron, metabolism of some amino acids, production of interferon, antiviral and antibacterial activity. Because of its in vivo inhibitory action on melanin synthesis, VC is useful as a whitening agent in cosmetics. However, l-ascorbic acid is less resistant to oxidative conditions than other vitamins and is easily decomposed. To solve this problem, we have tried to develop an ascorbic derivative that is stable in cosmetics, can be converted to l-ascorbic acid in the skin, and has sustained l-ascorbic acid efficacy in the skin. Numerous stable derivatives of VC have been developed. For example, magnesium-l-ascorbyl-2-phosphate (VC-PMG) is hydrolyzed to VC by phosphatase in the liver or skin. Studies have shown that VC-PMG suppresses melanin synthesis in B16 melanoma cells and cultured human melanoma cells. It is available as an additive in commercial cosmetics.

On the other hand, the rigid lipid lamellar structure of the stratum acts as a barrier, especially for the absorption of hydrophilic compounds. Therefore, chemical modification of the hydroxyl group of VC a lipophilic molecule is necessary for the development of an efficient transdermal ascorbic derivative. A variety of lipophilic ascorbic derivatives has been developed, but no available derivative is ideal in terms of stability, lipophilicity and biological activity.

Fatty acid esters of carbohydrates have been used as surfactants in cosmetics, detergents, and the food industry for a number of years. These compounds are typically non-ionic, non-toxic and biodegradable. Chemical alkylation of carbohydrates has been previously reported, and can be accomplished efficiently and inexpensively using base-catalyzed transacylation. Therefore, the development of alkylated ascorbyl phosphate may be suitable for improved skin penetration of ascorbyl phosphate.

Recently, we synthesized an amphiphilic ascorbic derivative, sodium isostearyl 2-O-1-ascorbyl phosphate (VCP-IS-Na), which exhibited lipophilic and satisfactory thermal stability. VCP-IS-Na has skin permeability superior to that of VC and sodium-2-O-1-ascorbyl phosphate (VCP-Na), and exhibits VC activity in vitro and in vivo after enzymatic hydrolysis to free VC by phosphatase and/or esterase.

In the present study, we examined the inhibitory effects of a novel amphiphilic ascorbic derivative, disodium isostearyl 2-O-1-ascorbyl phosphate (VCP-IS-2Na), which is more stable than VCP-IS-Na, on melanogenesis in cultured human melanoma cells, normal human melanocytes, and three-dimensional human skin models.

Experimental

General Procedure 1H- and 13C-NMR spectra (δ, J in Hz) were recorded on a JEOL GSX-500 NMR spectrometer. Tetramethylsilane was used as the internal reference (δ 0.00) for 1H-NMR spectra measured in chloroform-d6. This solvent was also used for 13C-NMR spectra. IR spectra were determined with a FT/IR-470 Pulse Fourier Transform Infrared Spec-
medium in 96-well plates. After 1 d of culture, the medium was changed to fresh medium containing various concentrations of VC, VCP-Na, and VCP-IS-2Na. HMV-II cells and NHEM were incubated for 10 d and 7 d, respectively, and then washed with PBS(−) and lysed with 100 μl/well of 0.1% Triton X-100/PBS(−). After mixing the lysates by vibration, 50 μl of 10 mM L-DOPA was added to each well. After incubation at 37 °C for 3 h, the absorbance was measured at 475 nm.

**Inhibitory Effect on Melanogenesis Using Cultured Human Skin Models**

The human skin model was placed in 6-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. After 1 h of culture, 50 μl of each sample solution at concentrations of 0.5% and 1% was applied to the surface of the tissue. The tissue was incubated for 3 weeks, and fed with 5 ml of fresh medium every other day.

**Determination of Melanin Content in Cultured Human Skin Models**

The human skin model was homogenized in 450 μl of 1% sodium dodecylsulfate (SDS) containing 0.05 mM ethylenediaminetetraacetate (EDTA), and 10 mM Tris HCl, pH 6.8. To each homogenate, 20 μl of 5 mg/ml protease K was added. After incubation at 45 °C for 10 min, the protease activity was determined by adding 50 μl of 500 mM sodium carbonate, and 10 μl of 30% hydrogen peroxide was added. Samples were maintained at 80 °C for 30 min. The mixture was extracted with 100 μl of chloroform : methanol (2:1) mixture. After centrifugation at 10000×g for 10 min, the optical density at 405 nm was determined. Synthetically melanin was used as a standard. MTT Assay of Cultured Human Skin Models The human skin model was placed in a 24-well plate, 300 μl of 1 mg/ml MTT (3-(4,5-dimethylthiazole-2-y1)-2,5-diphenyl tetrazolium bromide) solution was added to each well, and the tissue was incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO2 in air. After incubation, the tissues were washed with PBS(−), and 20 μl of isopropanol containing 0.04 M HCl was added to each well. The plate was shaken gently at room temperature for 2 h. The absorbance of extracts was measured at 570—660 nm.

**Statistical Analysis**

The proliferation, melanin content and tyrosinase activity data were expressed as the mean±standard error (S.E.), and subsequent inspection of means was evaluated by Student’s t-test between two groups at a significance level of p<0.05.

**Results**

**Effect of VCP-IS-2Na on Melanogenesis in HMV-II Cells**

To investigate the effects of VCP-IS-2Na on melanogenesis in human melanoma cells, HMV-II cells were cultured in medium containing various concentrations of VCP-IS-2Na. The melanin content of HMV-II cells was decreased by the addition of VCP-IS-2Na to the medium at concentrations of 100—300 μM (Fig. 2A). Melanin content was significantly inhibited at 300 μM, and the melanin content was reduced to 23% of that in non-treated cells. In contrast, VC and VCP-Na did not seem to inhibit melanin content at concentrations below 300 μM.

To exclude the possibility that the above inhibitory effects of VCP-IS-2Na on melanin synthesis might be caused by the inhibition of cell growth in human melanoma cells, we determined the number of cell grown of HMV-II cells, and the melanin content was decreased in a dose-dependent manner by the addition of VCP-IS-2Na to the medium at concentrations of 100—300 μM. These results indicate that VCP-IS-2Na had an inhibitory effect on melanogenesis at non-cytotoxic concentrations below 500 μM.

**Effect of VCP-IS-2Na on Melanogenesis in NHEM**

To investigate the effects of VCP-IS-2Na on melanogenesis in melanocytes, NHEM were cultured in medium containing various concentrations of VCP-IS-2Na. The melanin content of NHEM was decreased in a dose-dependent manner by the addition of VCP-IS-2Na to concentrations of 50—100 μM (Fig. 3A). Melanin content was significantly inhibited at 100 μM, and the melanin content was reduced to 52% of that in non-treated cells. In contrast, VCP-Na did not seem to inhibit melanin content at concentrations below 100 μM, while...
VC reduced melanin synthesis to 79% of that in non-treated cells at a concentration of 100 μM.

To exclude the possibility that the above inhibitory effects of VCP-IS-2Na on melanin synthesis might be caused by the inhibition of cell growth, we compared the number of NHEM grown in the presence and absence of VCP-IS-2Na (Fig. 3B). VCP-IS-2Na did not seem to inhibit the cell viability at concentrations below 100 μM. These results indicate that VCP-IS-2Na had an inhibitory effect on melanogenesis at non-cytotoxic concentrations below 100 μM.

Effects of VCP-IS-2Na on Cellular Tyrosinase Activity
To identify how melanin synthesis was inhibited, we examined the inhibitory action of VCP-IS-2Na on cellular tyrosinase activity in HMY-II cells and NHEM. As shown in Fig. 4A, treatment of HMY-II cells with VCP-IS-2Na at concentrations of 100—500 μM suppressed tyrosinase activity in a dose-dependent manner. VCP-IS-2Na inhibited 85% of tyrosinase activity at concentration of 500 μM in HMY-II cells. The 50% inhibition concentration (IC₅₀) value of VCP-IS-2Na was 314 μM in HMY-II cells. In contrast, VC and VCP-Na did not seem to inhibit tyrosinase activity at concentrations below 500 μM. Tyrosinase activity in NHEM was suppressed by the addition of VCP-IS-2Na at concentrations of 50—150 μM (Fig. 4B). VCP-IS-2Na inhibited 67% of tyrosinase activity at a concentration of 150 μM in NHEM. The IC₅₀ value of VCP-IS-2Na was 125 μM in NHEM. VCP-Na inhibited 43% of tyrosinase activity at a concentration of 150 μM, and VC slightly inhibited.

Effects of VCP-IS-2Na on Melanogenesis in Cultured Human Skin Models To identify the effects of VCP-IS-2Na on natural skin pigmentation, we used three-dimensional cultured human skin models. Figure 5 shows macroscopic darkening of a human skin model grown in the presence of different amounts of VCP-IS-2Na. Darkening of the model was clearly inhibited by the addition of 0.5% VCP-IS-2Na. When the cultured tissue was observed under an inverted microscope, there appeared to be fewer darkened melanocytes in VCP-IS-2Na-treated tissue than in non-treated tissue (Fig. 6). In contrast, macro- and microscopic observations of tissues treated with VC and VCP-Na did not appear to show fewer darkened melanocytes.

To quantitatively evaluate the inhibitory effect of VCP-IS-
2Na on cellular melanogenesis, the amount of melanin in the human skin models was measured (Fig. 7). With VCP-IS-2Na treatment at concentrations of 0.5% and 1.0% for 3 weeks, melanin production was significantly reduced to 30% and 21% of that in non-treated cells, respectively. The inhibitory effects of VC and VCP-Na on melanogenesis were significantly lower than that of VCP-IS-2Na.

Further, we evaluated cell viability in human skin models, which had been topically treated with different concentrations of VCP-IS-2Na, by MTT assay. No significant decrease in cell viability was observed when the tissue was treated with up to 1.0% of each sample (Fig. 8). These results suggest that topical treatment of tissue with VCP-IS-2Na decreased melanin synthesis without affecting cell viability.

**Discussion**

In the present study, the novel amphiphilic ascorbic derivative VCP-IS-2Na significantly inhibited the synthesized melanin content in cultured human melanoma cells, normal...
human melanocytes, and three-dimensional cultured human skin models. VCP-IS-2Na also significantly suppressed the cellular tyrosinase activity of cultured human melanoma cells and melanocytes. This decrease in the synthesized melanin content was well correlated with the inhibition of cellular tyrosinase activity in VCP-IS-2Na-treated melanoma cells and melanocytes. It has been suggested that the influence of VC on the monophase activity of tyrosinase results from its ability to reduce enzymatically generated O-quinones. Therefore, we conclude that VCP-IS-2Na directly or indirectly suppresses melanin synthesis catalyzed by human tyrosinase. Tyrosinase Related Protein-1 (TRP1) has been suggested to have a variety of catalytic functions including low levels of the enzymatic activities ascribed to tyrosinase.\(^{25,26}\) Our results indicated that VCP-IS-2Na directly or indirectly suppresses melanin synthesis catalyzed by human tyrosinase. Tyrosinase Related Protein-1 (TRP1) has been suggested to have a variety of catalytic functions including low levels of the enzymatic activities ascribed to tyrosinase.\(^{25,26}\) It has been reported that TRP1 possesses 5,6-dihydroxyindole-2-carboxylic acid oxidase activity, which can be blocked by VC. Moreover, VC may suppress melanin synthesis at various oxidative steps, such as 5,6-dihydroxyindole oxidation. Therefore, the inhibitory effects of VCP-IS-2Na on cellular tyrosinase activity may be due to the suppression of TRP1 activity with VC, which is released from VCP-IS-2Na.

We also evaluated the effect of VCP-IS-2Na on three-dimensional human skin models. Its inhibitory effect on melanogenesis was confirmed by quantitative measurements of melanin as well as macro- and microscopic observations of tissue pigmentation. Several researchers have indicated that human epidermal equivalents containing melanocytes and keratinocytes provide a convenient and reliable alternative to animal testing for evaluating the regulation of mammalian pigmentation.\(^{27,28}\) Our results indicated that VCP-IS-2Na affects melanin synthesis in these three-dimensional human skin models. Furthermore, this effect was observed without any detectable cytotoxicity to the human skin models.

In this study, it was difficult to compare the inhibitory effect of VCP-IS-2Na on the three-dimensional human skin models with that on human cells. When 500 µg of VCP-IS-2Na (50 µl of 1.0%) was applied to the tissue, the original concentration was approximately calculated as 19 mM, which is much higher than that applied to cells. However, the final concentrations of VCP-IS-2Na that permeated through the tissue into the medium were estimated to be much less than those of the applied VCP-IS-2Na. This may partly explain the difference in sensitivity between the three assay systems that we used. Further we are trying to characterize the inhibitory effects of VCP-IS-2Na on the expression of the tyrosinase gene and pigmentation in human skin.

VCP-IS-2Na inhibited melanin synthesis and tyrosinase activity in cultured human melanoma cells, normal human melanocytes, and three-dimensional human skin models to a greater degree than VC and VCP-Na. VCP-IS-2Na also significantly suppresses the cellular tyrosinase activity of cultured human melanoma cells and normal human melanocytes. Therefore, the inhibitory effect of VCP-IS-2Na on melanin synthesis is due to an inhibitory effect on cellular tyrosinase activity in human melanoma cells and normal human melanocytes. These results indicate that VCP-IS-2Na may be an effective whitening agent for the skin, and is metabolized from VCP-IS-2Na by phosphatase and/or esterase in the skin. Moreover, we expect the utilization of VCP-IS-2Na in the cosmetic industry.

**References**
