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Sodium dl-α-tocopheryl-6-*O*-phosphate inhibits PGE_2 production in keratinocytes induced by UVB, IL-1β and peroxidants

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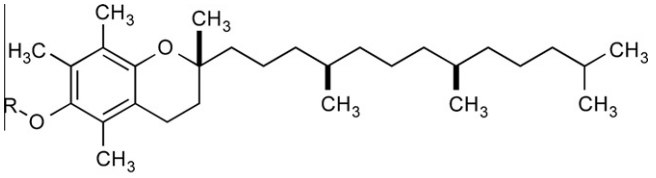
ABSTRACT

The water-soluble vitamin E derivative, sodium dl-α-tocopheryl-6-*O*-phosphate (**1**), exhibits protective effects against skin damage. As reported herein, we investigated the actions of **1** on the formation of the inflammatory mediator, prostaglandin E_2 (PGE_2), as compared to dl-α-tocopheryl acetate (**2**) and dipotassium glycyrrhizin acid (**3**). In a three-dimensional (3D) human skin model **1** was converted to α-tocopherol (Toc) to a greater extent than **2**. Post-treatment using 2% **1** following ultraviolet B (UVB) irradiation for 2 h significantly reduced photodamage as indicated by UVB-damaged cell formation and PGE_2 synthesis. In normal human epidermal keratinocytes stimulated with UVB irradiation, or exposed to interleukin-1beta, *tert*-butylhydroperoxide or hydrogen peroxide, pre-treatment with **1** (0–2 μM) inhibited PGE_2 production in dose-dependent manner to a greater extent than **2** and **3**. Increases in stimulator-induced cyclooxygenase 2 mRNA expression and p38 MAPK phosphorylation were suppressed by pre-treatment with **1**. The vitamin C derivative, magnesium L-ascorbyl-2-phosphate, significantly and synergistically, enhanced the inhibitory effects of **1** on PGE_2 production. These results suggest that **1** is a highly potent protective when compared among the examined commercial human skin care products, and that it might be useful for therapeutic and preventive medicine.

1. Introduction

Skin tissues are easily subjected to oxidative stress, in particular through the actions of reactive oxygen species generated by UV radiation. dl-α-Tocopherol (Toc) (Fig. 1), exhibits antioxidant effects, and it has been used to prevent and improve skin damage caused by free radicals. However, Toc is unstable due to its ease of oxidization. It is also highly hydrophobic, giving it oily characteristics that restrict its use in prescription drugs and cosmetic formulations.

In order to stabilize Toc, derivatives, have been developed that include esters of acetic acid and nicotinic acid. However, these derivatives retain oily or waxy characteristics that limit optimal pharmaceutical formulation. Consequently, a search for hydrophilic Toc derivatives led to the development of sodium dl-α-tocopheryl-6-*O*-phosphate (**1**), which bears a sodium phosphoryl group on the chroman ring of Toc (Fig. 1). Since **1** is water-soluble and stable against oxidation as compared with previous derivatives, it can be more easily applied to a variety of pharmaceutical formulations.



R = PO_3Na_2 **1**
R = $COCH_3$ **2**
R = H Toc

Figure 1. Chemical structures of **1**, **2** and Toc.

In skin tissues following transdermal administration, **1** permeates to the epidermis, where it is converted to Toc, which serves as a protectant against UV irradiation damage.[1] Compound **1** has also been reported to suppress telomere shortening that arises due to the instability of chromosome ends; a process that acts as a biological clock in mammalian cells.[2] Analogue **1** also promotes anti-proliferative and apoptosis-inducing activities in osteosarcoma (cancer) cells,[3] cardioprotective effects by ameliorating

Abbreviations: 1, sodium dl-α-tocopheryl-6-*O*-phosphate; 2, dl-α-tocopheryl acetate; 3, dipotassium glycyrrhizin acid; Toc, dl-α-tocopherol; PGE_2, prostaglandin E_2; HPLC, high-pressure liquid chromatography, UVB, ultraviolet B; IL-1β, interleukin-1beta; *t*BHP, *tert*-butylhydroperoxide; H_2O_2, hydrogen peroxide; LSE, living skin equivalent; Cox-2, cyclooxygenase-2; MAPK, mitogen-activate protein kinase.

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myocardial ischemic reperfusion injury,[4] and anti-atherosclerotic effects.[5] These actions are distinct from the anti-oxidative effects of Toc.[6] Recently, it has been found that trace amounts of **1** are present in vivo,[7,8,5] indicating that **1** may play physiological roles in addition to its anti-oxidant activity.

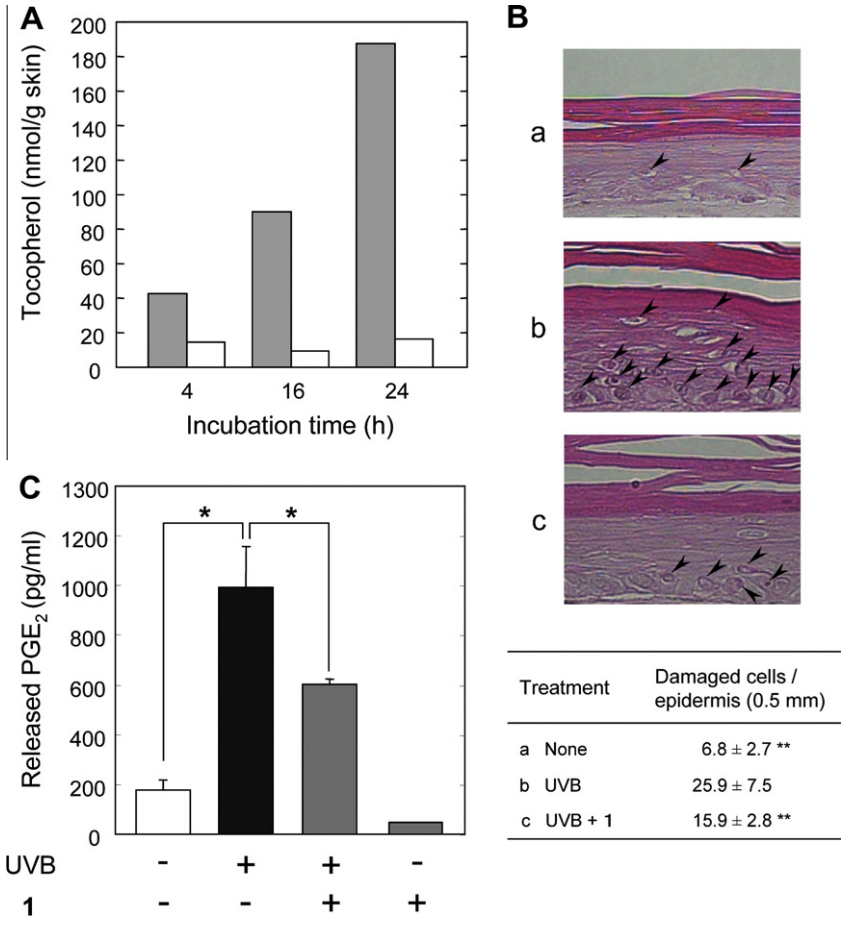
Prostaglandin E_2 (PGE_2) is a member of the PGE series that is well known as an inflammatory mediator that causes vasodilatation, hyperalgesia, and fever.[9] Numerous reports have appeared describing the relation between PGE_2 and UV inflammation, and that cutaneous UV-induced inflammation is mediated by PGE_2.[10] A marked increase in PGE_2 has been observed in the skin of rats,[11] mice[12] and humans[13] following UV irradiation. PGE_2 is released in keratinocytes in vitro in response to UV-irradiation.[14,15] Cyclooxygenase (Cox) converts arachidonic acid to PGH_2, a precursor of PGE_2.[16] There are two isoforms of Cox: one is constitutively expressed (Cox-1) and the other is inducible (Cox-2). Cox-2 is an immediate early gene that is induced by tumor promoters, growth factors, carcinogens, and inflammatory cytokines.[17] UV exposure increases the levels of Cox-2 expression in HaCaT cells and human skin.[18] The induction of Cox-2 expression by ultraviolet B (UVB) is mediated by p38 MAPK, an isoform of the MAPK family (ERK, p38, JNK etc.), that plays an essential role in UVB-induced elevation of Cox-2 promoter activity in human keratinocytes. Phosphorylation of cAMP-responsive element-binding protein and ATF-1 (activating transcription factor) is mediated by p38. In turn, these interact with the Cox-2 promoter.[19–21]

The effects of **1** have been previously studied on skin tissues subjected to UV irradiation.[1] However, it is not clear how potently **1** acts on skin tissues as a protective and ameliorative agent as compared with dl-α-tocopheryl acetate (**2**) or dipotassium glycyrrhizin acid (**3**), both of which are anti-inflammatory compounds used as quasi-drugs. It is also not clear whether **1** affects PGE_2 formation triggered by UVB, cytokines and peroxides, and by what mechanisms it might exert these effects. In the current study, using a three-dimensional (3D) reconstructed human skin model and normal human keratinocytes in vitro, we examined the effects of **1** on the amount of PGE_2 produced and the induction of Cox-2 in response to stimulants, in particular UV.

2. Results

2.1. Uptake and conversion of 1 to Toc in human model skin tissues

Initially, using a 3D restructured human skin model, we investigated whether uptake and conversion of **1** to Toc occur in skin



Treatment	Damaged cells / epidermis (0.5 mm)
a None	6.8 ± 2.7 **
b UVB	25.9 ± 7.5
c UVB + 1	15.9 ± 2.8 **

Figure 2. Protection of skin tissues by **1**. Experiments were performed using a 3D-restructured human skin model. (A) Permeation of **1** and **2** and conversion to Toc were determined. Solutions of compound **1** (■) or **2** (□) (2%, 100 μl) were applied onto the skin surface in the 3D-model. The skin was incubated at 37 °C for 8, 16, and 24 h and then homogenized. Intradermal concentrations of released Toc were determined by HPLC as described in the Section 4. Each point is the mean of at least three measurements. The SD of each point was ⩽8% of the mean. (B) Protective effects of **1** against UVB irradiation were examined. Specimens of the model skin irradiated by UVB (80 mJ/cm^2) without or with post-treatment using 2% of **1** were fixed and stained with 1% hematoxylin and eosin, and then UVB-damaged cells were counted under a microscope as described in the Section 4. Arrows indicate UVB-damaged cells. Values represent the mean of ±SD of each group. $^{**}p$ <0.01 versus UVB compared by Student's t test. (C) Suppressive effects of **1** on PGE_2 generation in skin are shown. Model skins were irradiated with UVB (80 mJ/cm^2) and then incubated without or with 2% of **1**. The amounts of PGE_2 in the medium were determined as described in the Section 4. Each bar represents the mean ± SD. $^{*}p$ <0.05 versus UVB irradiation in the absence of **1** compared by Student's t-test.

tissues, as compared with **2**. As shown in Figure 2A (gray bar), conversion of **1** to Toc was detected interdermally at 4 h following treatment with **1**. Toc concentrations increased in a time-dependent manner thereafter. The Toc concentration at 24 h was approximately 4.5-fold higher than at 4 h. The conversion of **2** to Toc at 4 h, was approximately two fifths of that from **1** (Fig. 2A, white bar). A time-dependent release of Toc from **2** was not observed with **1**. These results indicate that the release of Toc from **1** is more rapid than from **2**.

2.2. Protective and suppressive effects of post-treatment with 1 on PGE_2 formation in human model skin tissues caused by UVB irradiation

Next, using the 3D-reconstructed human skin model, we examined whether **1** could protect skin irradiated by UVB. Microtome sectioning was performed in a normal model of skin tissues in the absence of both UVB irradiation and treatment with **1**. These tests showed that smooth epidermis lacked appreciable UVB-damaged cells (Fig. 2B, a). In contrast, after UVB irradiation, epidermis did contain UVB-damaged cells, which stained darker than normal cells (Fig. 2B, b). Formation of UVB-damaged cell was also effectively suppressed by post-treatment with **1** (Fig. 2B, c). Damaged cells (darker cells) were counted under a microscope and quantified. As shown in Figure 2B, post-treatment with **1** significantly reduced the percentage of damaged cells resulting from UVB irradiation.

Since **1** appeared to protect skin from damage, we investigated the effects of **1** on skin tissue damage. These studies were performed in a model of UVB induced damage by measuring the amount of PGE_2 secreted into the medium. PGE_2 is a mediator of inflammation. Following UVB irradiation, the levels of released PGE_2 in skin tissues increased significantly, reaching approximately 5-fold higher levels as compared with non-UVB-irradiated skin (Fig. 2C). In contrast, after UVB irradiation, the secretion of PGE_2 from skin treated with **1** was significantly suppressed as compared to UVB-irradiated skin without **1** treatment (Fig. 2C). In addition, the use of **1** as a sole agent inhibited endogenous PGE_2 production (Fig. 2C). The enhancement of PGE_2 in response to UVB irradiation was suppressed by treatment with **1** following UVB irradiation. These results indicate that post-treatment of skin with **1** inhibits PGE_2 production caused by UVB irradiation, thereby protecting skin from inflammation.

2.3. Inhibitory effects of 1, 2 and 3 pre-treatment on PGE_2 formation in normal human epidermal keratinocytes (NHEK) stimulated with UVB irradiation, IL-1β or peroxides

In a 3D-model of skin tissue damaged by UVB irradiation, treatment with **1** following irradiation suppressed PGE_2 production.

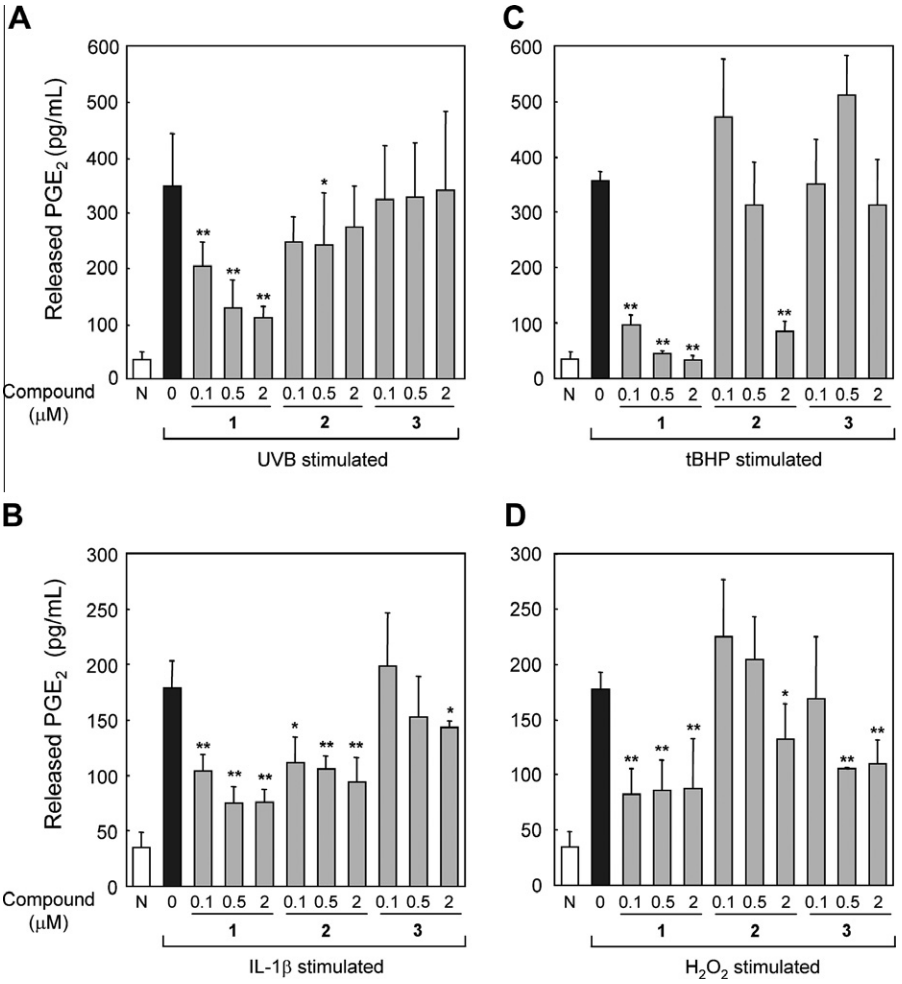


Figure 3. Effects of **1** on PGE_2 levels in NHEK. NHEK (10^5 cells/ml) were incubated with **1**, **2** and **3** at concentrations of from 0 to 2 μM for 24 h. (1) Cells were irradiated with UVB (60 mJ/cm^2) (A) or stimulated with IL-1β (10 ng/ml) (B) and then post-cultivated for 24 h as described in the Section 4. (2) After replacing the medium, cells were treated with 0.5 mM *t*BHP (C) or 1 mM H_2O_2 (D) for 0.5 h, transferred to fresh medium, and incubated for 24 h. The amounts of PGE_2 in the medium were determined as described in the Section 4. Each bar represents the mean ± SD. $^{**}p$ <0.01 and $^{*}p$ <0.05 versus each stimulant in the absence of compounds compared by Student's *t*-test.

This let us to examine whether pre-treatment with **1** in primary normal human epidermal keratinocytes (NHEK) could also affect PGE_2 formation induced by UVB irradiation as well as by the inflammatory triggers, interleukin-1beta (IL-1β), *tert*-butylhydroperoxide (*t*BHP), and hydrogen peroxide (H_2O_2). For comparison we used the known anti-inflammatory compounds, **2** and **3**. The levels of PGE_2 secreted from cells following UVB irradiation increased approximately 10-fold as compared to non-irradiated cells (Fig. 3A). Pre-treatment with **1** inhibited the increase in PGE_2 levels following UVB irradiation in dose-dependent manner (Fig. 3A). At concentrations of 0.1, 0.5 and 2 μM, **1** significantly suppressed PGE_2 secretion by approximately 44%, 65%, and 69%, respectively. In contrast, the inhibitory effects of **2** were much less than those of **1**, while **3** had hardly any effect (Fig. 3A).

The amount of PGE_2 secreted from IL-1β-stimulated cells increased approximately 5-fold as compared to normal cells (Fig. 3B). Pre-treatment with **1**, **2**, or **3** inhibited the amount of PGE_2 secreted from cells exposed to IL-1β in dose-dependent fashion (Fig. 3B). At concentrations of 0.1, 0.5 and 2 μM, **1** inhibited PGE_2 secretion by approximately 41%, 58%, and 57%, respectively. The inhibitory effects of **2** were also significant, being approximately 38% at 0.1 μM, 40% at 0.5 μM, and 67% at 2 μM, while **3** inhibited approximately 14% at 0.5 μM and 24% at 2 μM, which is much less than for **1** and **2**.

The amount of PGE_2 secreted from *t*BHP-treated cells, increased by approximately 10-fold as compared to normal cells (Fig. 3C). Pre-treatment with **1** inhibited the amount of PGE_2 secreted from cells treated with *t*BHP in a dose-dependent manner. At concentrations of 0.1, 0.5 and 2 μM, **1** inhibited PGE_2 secretion to the extent of approximately 74%, 87%, and 90%, respectively. In contrast, the inhibitory effect of **2** was approximately 77.4% at 2 μM. However, **3** did not significantly suppress the increase in PGE_2 levels, even at the highest concentration of 2 μM (Fig. 3C). When H_2O_2 was used as a peroxide, PGE_2 levels were suppressed approximately 50% by **1** (0.1–2 μM), 40% by **3** (0.5–2 μM), and 25% by **2** (2 μM) (Fig. 3D). Among the three tested compounds, **1** most effectively suppressed PGE_2 synthesis caused by UVB irradiation or by treatment with IL-1β or peroxides. Taken together, these results show that as compared with **2** and **3**, **1** is the most potent agent for inhibiting PGE_2 in NHEK following stimulation with a variety of triggers.

2.4. Effects of 1, 2 and 3 on Cox-2 mRNA expression in NHEK stimulated with UVB irradiation, IL-1β or peroxides

Cox-2 promotes inflammation and PGE_2 synthesis. We investigated whether Cox-2 mRNA in NHEK stimulated with triggers, was effected by pre-treatment with **1**, **2** or **3** under conditions that were similar to those show in Figure 3. Cox-2 mRNA expression in NHEK increased following UVB irradiation (1.6-fold) or treatment with IL-1β (1.2-fold), *t*BHP (4.7-fold) or H_2O_2 (3.3-fold) (Fig. 4, black bar) as compared to normal cells (Fig. 4, white bar). Compound **1** at 0.5 μM significantly inhibited the increase in Cox-2 mRNA expression stimulated by these triggers, (approximately 33% for UVB, 51% for IL-1β, and 23% for *t*BHP) with the exception of H_2O_2 (Fig. 4, gray bar). These results indicate that the protective effects of **1** might be due in part to inhibition of Cox-2 expression.

2.5. Effects of 1 on p38 MAPK activation in NHEK stimulated with UVB irradiation

It is known that members of the MAPK family play roles in chemical carcinogenesis by inducing Cox-2 gene expression,[22,23] and that UVB irradiation significantly increases p38 and ERK activities in cultured human keratinocytes.[24,25] An important role is played by p38 in signaling pathways associated with UVB-induced Cox-2 gene expression in human keratinocytes, whereas ERK may not be crucial in this process.[19] Consequently, we examined the effects of MAPK inhibitors (Bay 11-7082 for NF-κB; SB203580 for p38; SP600125 for JNK) on pathways involved in increasing PGE_2 levels in NHEK stimulated with UVB irradiation. We also examined whether **1** affects p38 phosphorylation.

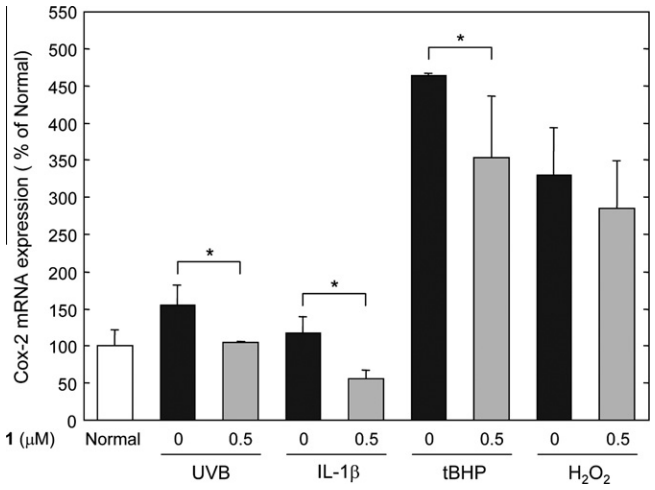


Figure 4. Suppressive effects of **1** on Cox-2 mRNA expression in NHEK caused by UVB irradiation, IL-1β and peroxides. NHEK (10^5 cells/ml) were incubated with **1** at concentrations of 0 and 0.5 μM for 24 h. After replacing with D-PBS (−) or medium lacking **1**, cells were irradiated with UVB (60 mJ/cm^2) or treated with 10 ng/ml IL-1β for 3 h, or 0.5 mM *t*BHP for 0.5 h or 1 mM H_2O_2 for 0.5 h, respectively. After stimulation, cells were post-cultivated for 3 h and collected. Cox-2 mRNA expression was determined by Real-time PCR methods as described in the Section 4. Each bar represents the mean ± SD. $^*p$ <0.05 versus each stimulant in the absence of **1** compared by Student's *t*-test.

The amount of PGE_2 secreted from UVB-treated cells increased approximately 2.7-fold as compared with normal cells (Fig. 5A). These amounts were reduced by pre-treatment with Bay 11-7082 (9%), SB203580 (73%), and SP600125 (33%). In particular, the p38 inhibitor SB203580 suppressed PGE_2 production to a greater extent than the other inhibitors. Under the same conditions, at concentrations of 0.1 and 0.5 μM, **1** significantly inhibited PGE_2 secretion in dose-dependent manner (Fig. 5B). These results indicate that suppression of PGE_2 production by **1** could be mediated by p38 MAPK pathways rather than by JNK MAPK or NF-κB pathways.

Next, we used anti-phospho-p38 and anti-p38 antibodies to measure the levels of phospho-p38 and p38 proteins in cells subjected to UVB irradiation, and we examined the effects of **1** on p38 phosphorylation. As shown in Figure 5C, UVB irradiation increased expression of phospho-p38 proteins. In contrast, treatment with 2 μM SB203580 or 0.5 μM of **1** significantly reduced these increases following UVB irradiation (approximately 62% or 55%, respectively). These results suggest that the inhibitory effects of **1** on PGE_2 synthesis might depend on inhibition of p38 phosphorylation. It should be noted that following pre-treatment of **1** for 24 h, the levels of **1** and Toc were 1.05 and 0.38 nmol/mg protein respectively (Fig. 5D), indicating that the actions of **1** might be due to both **1** and/or Toc.

2.6. Synergistic inhibitory effects on PGE_2 formation caused by UVB irradiation following pre-treatment with 1 in combination with post-treatment with a vitamin C derivative

Ascorbic acid (vitamin C) is a soluble anti-oxidant, which scavenges free radicals that destroy Toc. It does this by recycling Toc from an α-tocopheroxyl radical.[26,27] We examined whether post-treatment with the vitamin C derivative, L-ascorbyl-2-phosphate magnesium (APM) could enhance the reduction of PGE_2 formation in human keratinocytes (SVHKs) incurred by pre-treatment with **1**.

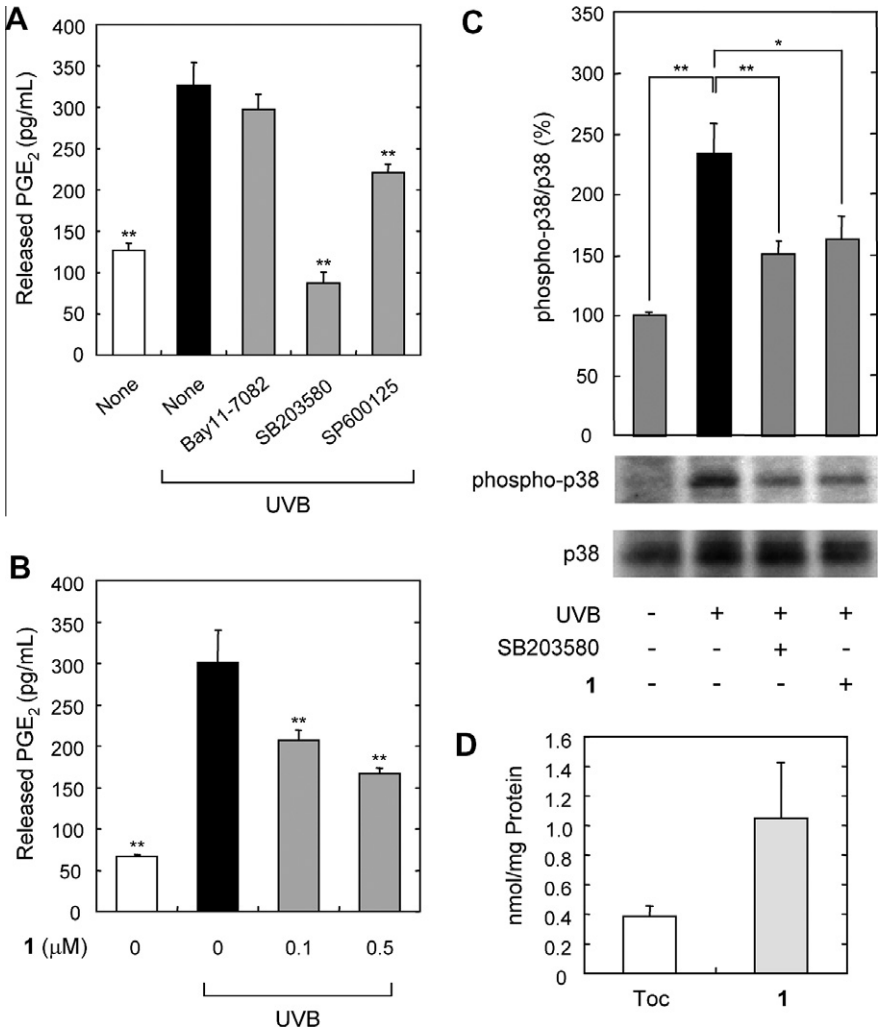


Figure 5. Suppression of p38 phosphorylation by pre-treatment with **1**. (A) NHEK (10^5 cells/ml) were incubated with Bay 11-7082, SB203580, or SP600125 at 2 μM concentration, and then irradiated with UVB (60 mJ/cm^2) as described in the Section 4. (B) NHEK (10^5 cells/ml) were incubated with **1** at the indicated concentrations, and then irradiated with UVB as described in the Section 4. The amounts of PGE_2 in the medium were measured. (C) NHEK (10^5 cells/ml) were incubated without/with 0.5 μM of **1**, and then treated without/with 2 μM SB203580. Cells were irradiated with UVB and post-cultivated for 0.5 h. Cellular proteins were prepared and analyzed by immunoblotting as described in the Section 4. (D) NHEK (10^5 cells/ml) were incubated with 0.5 μM of **1** and the amounts of **1** and Toc were analyzed by HPLC as described in the Section 4. Each bar represents the mean ± SD. $^{**}p$ <0.01 and $^{*}p$ <0.05 versus UVB irradiation in the absence of compounds compared by Student's *t*-test.

PGE_2 levels secreted from cells after UVB irradiation increased approximately 2-fold as compared to normal cells in the absence of UVB irradiation (Fig. 6, black bar). Under these conditions, the amount of PGE_2 secreted from UVB irradiated cells was reduced by approximately 0% and 13% following pre-treatment with 5 and 10 μM of **1** alone (Fig. 6, dark gray bar). No suppressive effects on PGE_2 secretion were observed in cells treated with 30 μM APM alone (Fig. 6, light gray bar). PGE_2 levels in cells treated with 5 and 10 μM of **1** in combination with 30 μM APM, were approximately 68% and 72% as compared to cells treated only with 5 and 10 μM of **1** (100%), respectively. Therefore, the addition of APM to cells treated with **1** significantly and synergistically reduced the generation of PGE_2 stimulated by UVB irradiation. PGE_2 levels in cells treated with 10 μM of **1** and 30 μM APM were nearly equivalent to normal cells in the absence of UVB irradiation. In SVHKs under similar conditions, no significant difference was observed between 0 μM of **1** and 5 or 10 μM of **1**. This was true whether or not APM was present. These results indicate that post-treatment with APM synergistically enhances the inhibitory effects on PGE_2 levels caused by pre-treatment with **1**. Since APM alone did not affect PGE_2 generation, APM could have a direct effect on actions of **1**, but not on the irradiation cascade.

3. Discussion

Compound **1** showed greater protective activity against markers of inflammation in skin tissues than **2** and **3**, which are well known anti-inflammatory agents. In a 3D-human skin model, post-treatment with **1** suppressed both UVB-damaged cell formation and PGE_2 production induced by UVB irradiation (Fig. 2). In addition, in NHEK, pre-treatment with **1** inhibited to a greater extent than **2** and **3** the production of PGE_2 induced by UVB irradiation and the inflammatory triggers, IL-1β, *t*BHP and H_2O_2 (Fig. 3). Cox-2 mRNA expression (Fig. 4) and p38 phosphorylation (Fig. 5), were also suppressed by pre-treatment with **1**, in good correlation with the suppression of PGE_2 production. The inhibitory activity of **1** on PGE_2 production was enhanced when combined with APM (Fig. 6). From this data it can be concluded that **1** exhibits an ability to inhibit PGE_2 production induced by various proinflammatory factors mediated through Cox-2 expression and

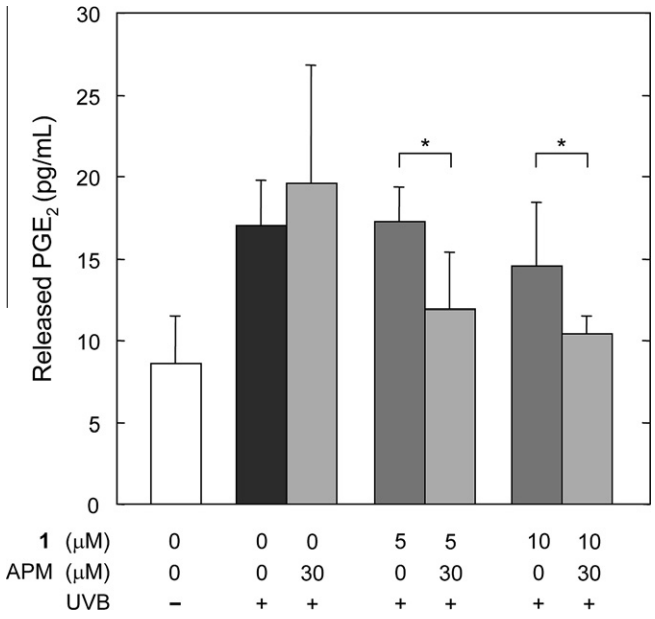


Figure 6. Synergistic effects of **1** in combination with APM on PGE_2 synthesis in human keratinocytes caused by UVB irradiation. Human keratinocytes (SVHKs, 5×10^4 cells/ml) were incubated with **1** at concentrations of 0–10 μM for 24 h. After replacing to D-PBS(−), cells were irradiated with UVB (30 mJ/cm^2). Cells were post-cultivated for 24 h in medium with or without 30 μM APM, and the amounts of PGE_2 secreted into the medium were determined by ELISA methods as described in the Section 4. Each bar represents the mean ± SD. $^*p$ <0.05 versus UVB irradiation done in the absence of APM with **1** treatment compared by Student's *t*-test.

p38 phosphorylation. In addition, **1** suppresses PGE_2 levels synergistically in cooperating with APM. In the current study, we shed new light on issues related to the effects of **1** on inflammatory mediators and PGE_2 generation, and we compare the potency of **1** with other anti-inflammatory agents.[1] Our data show that **1** is the most effective agent among those examined in suppressing PGE_2 production, Cox-2 expression and p38 phosphorylation.

It has been reported that pre-treatment with **2** inhibits PGE_2 production[28] in skin tissues of hairless mice irradiated with UVB. However, this is not observed following post-treatment. In addition, Cox-2 expression in mouse skin tissues irradiated with UVB is suppressed by pre-treatment with **2**. In the current study, **2** exhibited a significant ability to suppress PGE_2 synthesis in NHEK induced by UVB irradiation (Fig. 3A). These results are in agreement with previous reports, although there are differences between in vivo results using mouse skin and in vitro results using normal human keratinocytes. Our data also show that **1** is a more effective anti-inflammatory agent than **2**.

In Chinese medicine, **3** is used as an effective anti-inflammatory agent.[29,30] Several biological activities of **3** have been identified in the human body, including anti-hyperlipidemic, anti-oxidative,[31] anti-viral[32] and interferon-inducing[33] effects. However, the biochemical mechanisms of action of **3** remain to be elucidated.[34–37] It has been reported that **3** inhibits in a dose dependent manner, PGE_2 production in activated rat peritoneal macrophages at concentrations higher than 10 μg/ml (12.1 μM).[34] Our current study examines for the first time the effects of **3** on PGE_2 levels in human skin tissues stimulated by UVB irradiation as well as by IL-1β, *t*BHP and H_2O_2. Compound **3** (2 μM) inhibited PGE_2 production in NHEK induced by IL-1β and H_2O_2, but not by UVB or *t*BHP (Fig. 3). It was also less potent than **1** in inhibiting the induction of PGE_2 synthesis (Fig. 3).

The MAPK family of serine/threonine protein kinases, have been shown to be important regulators in signaling pathways leading to proto-oncogene expression.[38–40] Since Cox-2 expression plays an important role in UV carcinogenesis, p38 may represent a potential molecular target for chemoprevention of skin cancer. In our current study, **1** is more potent than **2** in the suppression of PGE_2 synthesis stimulated by UVB, IL-1β, *t*BHT and H_2O_2 (Fig. 3). Compound **1** also suppressed Cox-2 expression (Fig. 4) and p38 phosphorylation (Fig. 5). These results indicate that **1** inhibits PGE_2 synthesis via Cox-2 expression through p38 phosphorylation. Accordingly, **1** potentially could be useful as an anti-inflammatory and cancer preventive agent.

Suppression of lipid peroxidation in cell membranes is a major function of Toc. Ascorbic acid in the cytosol directly scavenges free radicals species that could otherwise destroy Toc, and it recycles Toc from α-tocopheroxyl radical.[26,27] In primary cultures of hepatocytes[41,42] or dermal fibroblasts,[43] initial concentrations of both Toc and ascorbate progressively decrease with time. The cause of this reduction has been attributed to oxidative stress.[41] In cultured H4IIE rat liver cells, ascorbate supplements preserve Toc, and both ascorbate and Toc supplements decrease lipid peroxidation in cell membranes.[44] These results indicate that ascorbate loading of cells spares cellular Toc either directly or through recycling of Toc by preventing lipid peroxidative damage due to oxidative stress. In the current study, APM synergistically enhanced the inhibition of induced PGE_2 synthesis by **1** (Fig. 6). This effect may either be due to recycling of Toc derived from **1** directly or by APM. In conclusion, the results of the current study show that **1** is an excellent inhibitor of inflammatory mediators in human skin tissues and that it serves as a protective agent against exogenous stimulants. Compound **1** is chemically stable and potentially it could be useful as a provitamin E supplement or as a sole agent for human skin protection and skin cancer prevention.

4. Experimental

4.1. Chemicals and materials

Compound **1** was purchased from Showa Denko Co. Ltd (Tokyo, Japan). *t*BHP and H_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Compounds **2**, **3**, SB203580 and SP600125 were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The purities of **1** and **2** were greater than 98%, and the amount of Toc and other Toc derivatives in **1** and **2** was less than 2%. Bay11-7082 was obtained from Calbiochem Inc. (San Diego, CA, USA), and antibodies against p38 MAPK and phospho-specific p38 MAPK were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals were of reagent grade.

4.2. 3D-human reconstructed skin culture and UVB irradiation

Living skin equivalent (LSE) (TESTSKIN-LSE high, Toyobo, Co. Ltd, Osaka, Japan), which is a 3D-reconstructed human skin model, was incubated in the LSE assay medium (Toyobo) at 37 °C in a humidified atmosphere of 5% CO_2 in air according to the manufacturer's protocol. The epidermal side of LSE was irradiated with UVB at a range of 80 mJ/cm^2, using a UV Crosslinker (CL-1000M, middle wavelength at 302 nm, UVP LLC, Upland, CA, USA), and added the solution without or with 2% of **1** on its surface, and incubated at 37 °C for 2 h. After the solution containing **1** was removed and fresh medium was added, the skin models were incubated for 22 h. The amounts of PGE_2 were determined by ELISA methods.

4.3. Histochemical analysis of UVB-damaged cell formation

Skin samples of LSE were fixed with 10% formalin neutral buffer solution. For microscopy, specimens were dehydrated with ethanol and embedded in paraffin, and were then stained with 1% hematoxylin and eosin. Photographs of these sections were examined

and counts made of the number of UVB-damaged cells, which were distinguished by their dense, dark-staining (darker than neighboring keratinocytes), irregular nuclei in 0.5 mm of epidermis at 8 or 12 sites along the culture skin.

4.4. Cell culture

Normal human epidermal keratinocytes (NHEK) (Kurabo, Osaka, Japan) were propagated in HuMedia-KG2 (Kurabo). SV40-transformed human keratinocytes (SVHKs) were kindly supplied by Professor Shingo Tajima in National Defense Medical College, and were propagated in Dulbecco's modified medium (Sigma) containing 10% fetal bovine serum (FBS).

4.5. UVB irradiation and stimulation with IL-1β or peroxides

NHEK (10^5 cells/ml) grown in HuMedia-KG2 were incubated with various concentrations of **1**, **2**, and **3** for 24 h, and washed with Dulbecco's phosphate-buffered saline Ca^{2+}, Mg^{2+} free (D-PBS(−), Wako). *UVB*: Cells were irradiated with UVB at an intensity of 60 mJ/cm^2 using a UV Crosslinker (UVP LLC), and then washed with D-PBS(−) and placed in fresh medium prior to incubating at 37 °C for 24 h. *IL-1β*: Cells placed in fresh medium were treated with 10 ng/ml IL-1β for 24 h at 37 °C. *Peroxides*: Cells placed in fresh medium were treated with 0.5 mM *t*BHP for 0.5 h or 1 mM H_2O_2 for 0.5 h at 37 °C, washed with D-PBS(−), suspended in fresh medium, and then incubated at 37 °C for 24 h. Each medium was used for the determination of PGE_2 levels.

4.6. PGE_2 assay

PGE_2 levels in medium were determined by an enzymatic immunoassay kit (Cayman Chemicals, MI, USA) according to the manufacture's instructions.

4.7. Determination of Toc content

LSE samples or NHEK (10^6 cells) were homogenized in 0.5 ml of 50 mM HEPES buffer (pH 7.2), using a sonicator (Ohtake works, Tokyo, Japan). Homogenates (1.5 mg/ml, 0.4 ml) were transferred to a screwcap tube to which 0.1 ml of 10 nmol/ml δToc in ethanol, 0.5 ml of 5% pyrogallol in ethanol, and 0.05 ml of concentrated HCl were added, and the solution was mixed vigorously for 30 second. The homogenates were extracted with 2.5 ml of *n*-hexane/acetic acid ethyl ester (9:1), and then centrifuged. The upper layer was collected and dried using a centrifuged concentrator, and then the residue was diluted with 200 μl of high-performance liquid chromatography (HPLC) solvent. After filtration, 10 μl of the mixture was applied to an HPLC column. The concentrations of endogenous Toc were determined by HPLC using a reverse-phase column (C18M 4E, 5 mm, 4.6 × 250 mm, Showa Denko Co. Ltd, Tokyo, Japan). HPLC was performed using a Shimadzu HPLC system (LC20, Shimadzu, Kyoto, Japan) with a mobile phase consisting of 7:3 (v/v) of methanol/acetonitrile containing 0.03 M CH_3COOH and 0.03 M CH_3COONa at a flow rate of 1.0 ml per min at 4 °C. The fluorescence intensities for Toc and **1** were monitored at excitation 290 nm and emission 325 nm. Retention times for **1** and Toc were 7 and 13 min, respectively.

4.8. Real-time PCR analysis

NHEK in HuMedia-KG2 were incubated without and with 0.5 μM of **1** for 24 h, and washed with D-PBS(−). Cells were cultivated for 3 h after UV irradiation or the incubation with triggers (10 ng/ml IL-1β (3 h), 0.5 mM *t*BHP (0.5 h), 1 mM H_2O_2 (0.5 h)) as described above, harvested, and frozen in RNAcell protect reagent (QIAGEN, Valencia, CA, USA). Total RNA was isolated from cells by RNeasy Plus Mini (QIAGEN). Single-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit (Perfect Real Time, TAKARA BIO INC., Shiga, Japan). Real-time PCR was performed using the Roche LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with SYBR Premix Ex TaqII (TAKARA BIO INC.) and primers specific for the Cox-2 genes and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) (Primer set ID: HA033050 for Cox-2, HA031578 for GAPDH, TAKARA BIO INC.). A single fluorescence reading at 530 nm was obtained for each sample at the extension step. Samples were analyzed using LightCycler software.

4.9. Western blotting analysis

NHEK in HuMedia-KG2 were incubated without and with 0.5 μM of **1** for 24 h, and washed with D-PBS(−). Cells in medium were incubated with the respective inhibitors, Bay 11-7082 (for NF-κB), SB203580 (for p38 MAPK), or SP600125 (for JNK MAPK) at 2 μM concentration for 1 h. The reagents were used at a concentration of 2 μM, which has no influence on cell survival rate. After removing the medium and washing with D-PBS(−), cells were subjected to UV irradiation, replaced in fresh medium and incubated for 0.5 h. Cells were harvested and lysed using Buffer A (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, phosphatase inhibitor cocktail, and protease inhibitor cocktail). After centrifugation (13,000×*g*, 20 min), the supernatants were separated by 10% SDS–polyacrylamide gel electrophoresis and immunoreactivity with primary antibody was demonstrated using the ECL plus Western Blotting System (General Electric Co., U.K.).

4.10. Compound 1 in combination with APM

SVHKs (5×10^4 cells/ml) grown in Dulbecco's modified medium containing 10% FBS were incubated with **1** at the concentrations of 0 to 10 μM for 24 h. After replacing to D-PBS(−), cells were irradiated by UVB (30 mJ/cm^2). Cells were post-cultivated for 24 h in medium containing 0.5% FBS with or without 30 μM APM, and the amounts of PGE_2 in the medium were determined as described above.

4.11. Statistics

The statistical significance of the data was evaluated by the Student's *t* test. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 were considered significant. Each experiment was performed at least 3 times and repeated.

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