**A Thesis for the Degree of Master of Science**

# 20(*S*)-Protopanaxadiol, an Aglycone Derivative of Ginsenoside, Inhibits Solar Ultraviolet-induced Matrix Metalloproteinase-1 Expression

# 인삼 대사체 20(*S*)-protopanaxadiol의 자외선에 의한 피부주름 개선효능 및 작용기작 규명

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**By**

**Seungmin Han**

**Department of Agricultural Biotechnology**

**Seoul National University**

# ABSTRACT

Ginseng is widely used as traditional medicinal plant in Korea and it has been reported various biological activities such as anti-tumor and anti-diabetic. Among its potential health benefits, ginseng has shown positive effects on preventing skin aging caused by ultraviolet (UV) irradiation. Ginsenosides are the major pharmacologically active components of ginseng and 20-*O*-*β*-*D*-glucopyranosyl-20(*S*)-protopanaxadiol (GPPD) and ginsenoside Rb1 (Rb1) were reported to have anti-wrinkle effects. In this research, 20(*S*)-protopanaxadiol (20(*S*)-PPD), an aglycone derivative of Rb1 metabolite was focused on its potential anti-wrinkle benefit compared to other ginsenosides, GPPD and Rb1. Anti-wrinkle effect of 20(*S*)-PPD against solar UV was investigated in human skin equivalent and human keratinocytes. 20(*S*)-PPD strongly attenuated solar UV-induced matrix metalloproteinase (MMP)-1 expression compared to GPPD and Rb1. It was found that 20(*S*)-PPD regulated

MMP-1 protein expression and activator protein (AP)-1 gene transcriptional activity. In addition, 20(*S*)-PPD suppressed both in MEK1,2/ERK/P90$^{RSK}$ and MEK3,6/p38 pathways. These signaling pathways stimulate transcription factor AP-1 which regulate MMP-1 expression. Taken together, these findings suggest that 20(*S*)-PPD could be a potential agent for preventing solar UV-induced skin wrinkle.

**Keywords: 20(*S*)-protopanaxadiol; Wrinkle; MMP-1; Solar UV**

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# CONTENTS

# Ⅰ. INTRODUCTION

The skin is one of the largest organs in the human body and most exposed surface to the environment [1]. Skin barrier affects and has links with environment and our body [2]. Skin changes consist of clear evidence of the aging process. Skin aging is influenced by several factors, and these are classified intrinsic and extrinsic factors [3]. Among the extrinsic factors, solar ultraviolet (UV) irradiation is of considerable importance for skin aging. Skin aging due to UV exposure, called photoaging, is superimposed on chronological skin aging [4]. In addition, about 80 % of facial skin aging is mainly due to solar UV [5]. Major distinctive symptoms of photoaged skin is wrinkles, dry, laxity, rough pigmentation, and a leathery [4, 6].

Solar UV is divided into three subgroups including UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm) [7]. Since

Ozone layer effectively filter out UVC, UVA and UVB are able to reach the human skin [8, 9]. Solar UV which is consisted of 95% UVA and 5% UVB is an actual cause of skin wrinkle [10].

Exposure to solar UV results in the increase of matrix metalloproteinases (MMPs) which degrade skin collagen and may play a role in wrinkle formation by photoaging process [11]. Repeated exposure to solar UV alters biological pathways, which decrease procollagen synthesis and break down collagen-rich extracellular matrix (ECM) by overexpressing MMPs [11, 12]. Certain MMPs such as MMP-1 (collagenase), MMP-2 (gelatinase), and MMP-9 (gelatinase) are mostly expressed in the human skin [2, 13, 14]. Among those MMPs, MMP-1 plays the most biologically important role in the degradation of the type Ⅰ and Ⅲ collagens which consist of ECM [2, 9, 15]. Therefore, attenuating collagen degradation through inhibition of MMP-1

expression can be one of the great therapeutic strategies for anti-wrinkle effect.

Ginseng refers to as the root of Panax ginseng C.A. Meyer and it is widely used as the most valuable traditional medicinal plants particularly in Korea, China and Japan [16, 17]. Pharmacological efficacy of ginseng has been reported such as anti-tumor activity [18], anti-diabetic efficacy [19], improving liver function [20], and anti-oxidation activity [21]. Among their potential health benefits, Ginseng extract can help to prevent skin wrinkle caused by UV irradiation [22-25].

Ginsenosides, natural product steroid glycosides and triterpene saponins are the major pharmacologically active constituents of ginseng [26-28]. It have been the target of a lot of study considered to be the major active compounds behind the claims that ginseng has pharmacological properties [22-24]. Up to date, more than 150 naturally

derived ginsenosides have been isolated from various parts of ginseng [28]. Orally administered ginseng extract is metabolically converted to various ginsenoside such as ginsenoside Rb1 (Rb1, Fig. 1A), 20-*O*-*β*-*D*-glucopyranosyl-20(*S*)-protopanaxadiol (GPPD, Fig. 1B), and 20(*S*)-protopanaxadiol (20(*S*)-PPD, Fig. 1C) by intestinal bacteria [29]. Previous literatures have demonstrated the beneficial activities of ginsenosides on the skin health, such as whitening [30, 31], hydration [32] and anti-wrinkle effects [33-35]. In particular, the anti-wrinkle effect of GPPD and Rb1 were reported [36, 37]. However, the anti-wrinkle effect and mechanism of 20(*S*)-PPD, an aglycone derivative of Rb1 metabolite has not been investigated yet.

In this study, I elucidated the anti-wrinkle effect of 20(*S*)-PPD on solar UV-induced MMP-1 expression in human skin equivalent and human keratinocytes (HaCaT). I also sought to investigate the molecular mechanisms responsible for preventing UV-induced skin

wrinkle. These findings indicate that 20(*S*)-PPD could be used as a potential therapeutic anti-wrinkle agent.

# Ⅱ. MATERIALS AND METHODS

## 2.1. Chemicals and reagents

20(*S*)-PPD and GPPD were provided by Ambo institute (Seoul, Korea). Rb1 was provided by Naging zelang medical technology Co., Ltd (Jiansu, China). Dulbecco's modified eagle medium (DMEM) was purchased from Hycolne (Long, UT). Fetal bovine serum (FBS) was bought from Sigma-Aldrich (St.Louis, MO). Penicillin-Streptomycin Solution was purchased from Mediatech, Inc. (Manassas, VA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega Corporation (Madison, WI). The MMP-1 antibody was obtained from R&D systems Inc. (Minneapolis, MN). Antibodis against phosphorylated extracellular-signal regulated kinas 1/2 (ERK1/2) (Thr202/Tyr204), total ERK1/2, total c-Jun N-terminal kinas 1/2 (JNK1/2), phosphorelated-p38 (Thr180/Thr182), and total p38

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA).

### 2.2. Cell Culture and treatments

HaCaT cells were purchased from CLS (CLS Cell Lins Services GmbH, Heiderberg, Germany). HaCaT cells were cultured in DMEM with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin at 37 ℃ and 5 % $CO_2$.

### 2.3. Solar UV irradiation

HaCaT cells were exposed to solar UV at 25 kJ/$m^2$ in serum free media. Human skin equivalents were exposed to solar UV at 30 kJ/$m^2$ in serum free media. Solar UV resource was purchased from Q-Lab Corporation (Cleveland, OH). The UVA-340 lamps were purchased

in the best possible simulation of sunlight in the critical short wavelength region from 365 nm down to the solar cutoff of 295 nm with a peak emission of 340 nm. The percentage of UVA and UVB of UVA-340 lamps was measured at a UV meter and was 94.5 % and 5.5 % respectively.

**2.4. Cell viability**

The cell cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. HaCaT cells were cultured in the 96 well plates at a density of $10\times10^4$ cells/well and incubated in DMEM-10% FBS contacting penicillin/streptomycin at 37 ℃ and 5 % $CO_2$. Cell were starved in serum-free DMEM for 24 h. The cells were treated with or without various concentrations of samples for 1 h, followed by solar UV at 25 kJ/$m^2$. The cells were incubated for 48 h after solar UV irradiation

at 37 ℃ and 5 % $CO_2$. After then, Cells were treated with MTS solution which activated by PMS solution to 2 h. The absorbance at 490 nm was measured by using a microplate reader (Molecular Devices, CA).

**2.5. Preparation of human skin equivalent**

I purchased Neoderm® -ED which is the human skin equivalent from TEGO Science (Seoul, Korea). Briefly, Human dermal fibroblasts (HDFs) were cultured in the collagen matrix for 1 day. And then keratinocytes were seeded on top of collagen matrix and co-cultured for 4 days. Next, lifted the keratinocytes and HDF block to expose the air. And 20(S)-PPD was treated for 1 h after 7 days of the air-lift. After that, human skin equivalents were irradiated 30 $kJ/m^2$ solar UV twice a day for 2 days. During 2 days human skin equivalents are incubated at 37 ℃ and 5 % $CO_2$.

### 2.6. Masson's trichrome staining and immunohistochemistry

To evaluate collagen in the dermis, Masson's trichrome staining was performed. Human skin equivalents were fixed with 10 % neutral-buffered formalin, and embedded in paraffin. Serial sections (4 μm) were mounted onto slides. After deparaffinizing, skin sections were stained with Hematoxylin for 5 min. And then, slides were washed and stained in biebrich scarlet and acid fuchsin. Next, the slides were placed in phosphomolybdic-phosphotungstic acid for 10 min and aniline blue for 5 min to stain collagen. Slides were then washed and incubated in 1 % acetic acid for 15 min. Lastly, they were dehydrated and washed. Skin sections were examined at 400× magnification using an Olympus AX70 light microscope (Tokyo, Japan). To measure the level of MMP-1, human skin equivalents were fixed, embedded, and deparaffinized as described above. Slides were incubated in 0.3 % hydrogen peroxide and blocked using 5 % normal goat serum for 30 min. After blocking, slides were

incubated with MMP-1 antibody (Santa Cruz, CA) at 4 ℃ overnight. Next, they were reacted to biotinylated secondary antibody (Vector Labs, Burlingame, CA) and developed using avidin-biotin complex kit (Vector Labs). The reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride hydrate solution (Vector Labs). And the counterstain was conducted using hematoxylin. The level of MMP-1 in human skin equivalent sections was examined under 400× magnification using an Olympus AX70 light microscope.

**2.7. Western blot**

HaCaT cells were cultured for 48 h, and then were incubated in serum-free DMEM for 24 h. After that, the cells were treated with or without various concentrations of 20(*S*)-PPD, GPPD, and Rb1 (0.25, 0.5 and 1 μg/ml) for 1 h, followed by solar UV (25 kJ/$m^2$) irradiation. The media was harvested on ice, and then centrifuged at 18,620 g for 10 min.

The protein concentration was measured using a protein assay reagent kits as described by the manufacturer. The proteins were separated electrophoretically using a 10 % SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (MERK Millipore). The membrane was blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 ℃ overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, London, UK) after hybridization with the HRP-conjugated secondary antibody (Life technologies). Western blot data were quantified using the program Image J (NIH, USA).

**2.8. Gelatin zymography**

Gelatin zymography was performed in 12 % polyacrylamide gel in the presence of gelatin (0.1 % w/v) as a substrate for MMP-2. The protein samples were mixed with loading buffer (10 % SDS, 25 %

glycerol, 0.25 M Tris (pH 6.8) and 0.1 % bromophenol blue), and then run on 12 % SDS-PAGE gel without denaturation. Afterward, the gel was washed with renaturing buffer (Life technologies) for 1 h at room temperature and incubated for 24 h at 37 ℃ in developing buffer (Life technologies). After enzyme reaction, the gel was stained with 0.5 % Coomassie brilliant blue in 10 % acetic acid.

**2.9. Real-time quantitative PCR**

HaCaT cells were treated with 20(S)-PPD for 24 h and harvested in RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After RT with oligo-dT primers using a PrimeScript$^{TM}$ 1$^{st}$ strand cDNA synthesis Kit (Takara Bio Inc.), Real-time quantitative RT-PCR was performed using IQ SYBR (Bio-Rad Laboratories) and 2 μl of cDNA in triplicate with Glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) as internal control. Before PCR amplification, the primers were denatured at 95 ℃ for 3 min. Amplification was made up of 44 cycles at 95 ℃ for 10 sec, 60 ℃ for 30 sec, and 72 ℃ for 30. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was probed by the following primer: MMP-1 forward (5'-CCC CAA AAG CGT GTG ACA GTA-3'); MMP-1 reverse (5'-GGT AGA AGG GAT TTG TGC G-3'); GAPDH forward (5'-GAG TCA ACG GAT TTG GTC GT-3'); GAPDH reverse (5'-TTG ATT TTG GAG GGA TCT CG-3').

**2.10. Luciferase reporter gene assay**

The lentiviral expression vectors, including pGF-AP1-mCMV-EF1-Puro (System Biosciences, CA), and packaging vectors, including pMD2.0G and psPAX, were purchased from Addgene Inc

(Cambridge, MA). pGF-AP1-mCMV-EF1-Puro vector and the packaging vectors (pMD2.0G and psPAX) were transfected into HEK293T cells using jetPEI following the manufacturer's instructions. The transfection medium was changed at 24 hours after transfection and the cells were then cultured for 36 hours. The viral particles were harvested by filtration using a 0.45-mm syringe filter, then combined with 8 μg/mL polybrene (EMD Millipore) and infected into 60% confluent HDF cells overnight. The cell culture medium was replaced with fresh complete growth medium for 24 hours before the cells were selected for using puromycin (Sigma, MO, 2 μg/mL) over 36 hours. The selected cells were then used for further experiments. HaCaT cells were cultured for 48 h and then starved in serum-free DMEM for 24 h. After starvation, the cells were treated with or without various concentrations of 20(S)-PPD for 1 h, followed by 25 kJ/$m^2$ solar UV irradiation. Cell extracts were prepared with reporter lysis buffer

(Promega), and the extracts were used for luciferase assay. MMP-1 and activator protein (AP)-1 activity in HaCaT cells were determined by using a luciferase assay kit (Promega), as described by the manufacturers.

### 2.11. Statistical analysis

Differences between control and solar UV-irradiated control were assessed with Student's t-test. To compare the difference between the solar UV-exposed groups, one-way ANOVA was used with Duncan. The data were statistically analyzed with IBM SPSS statics ver. 23.0 (IBM Co., Armonk, NY, USA) and $p$ values of less than 0.05 were considered statistically significant.

# Ⅲ. RESULTS

### 3.1. 20(*S*)-PPD inhibits solar UV-induced MMP-1 protein expression in HaCaT cells.

To verify the anti-wrinkle effect of 20(*S*)-PPD, I evaluated the inhibitory effects on solar UV-induced MMP-1 protein expression in HaCaT cells compare to Rb1 and GPPD. As a result, 20(*S*)-PPD showed the greatest inhibitory effect among them. This result suggests that 20(*S*)-PPD inhibits solar UV-induced MMP-1 protein expression in HaCaT cells (Fig 1A, B).

# Figure 1

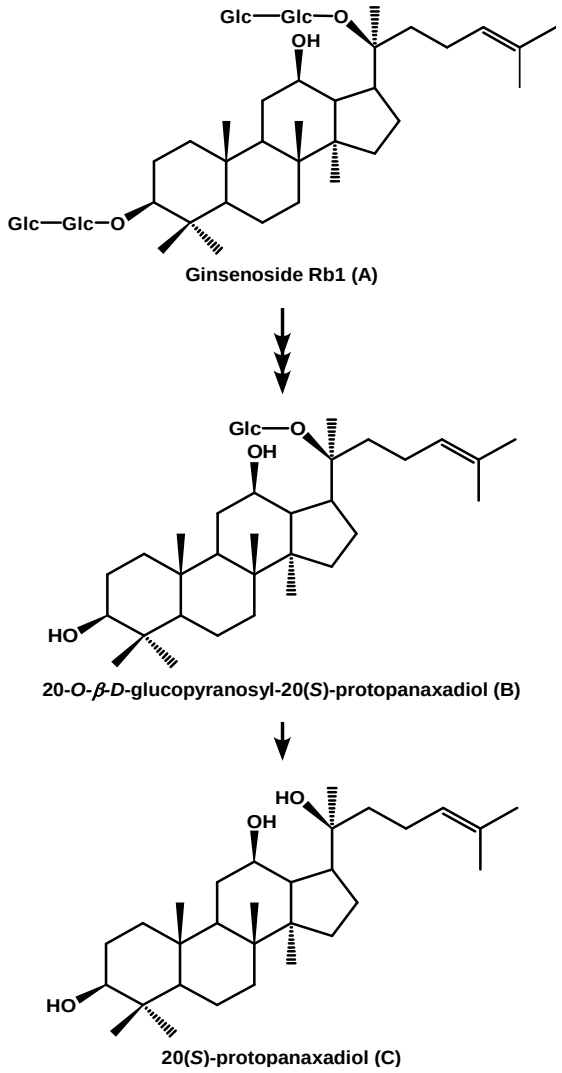


**Figure 1. Chemical structure and bioconversion of ginsenoside Rb1 (Rb1), 20-*O*-*β*-*D*-glucopyranosyl-20(*S*)-protopanaxadiol (GPPD) GPPD, and 20(*S*)-protopanaxadiol (20(*S*)-PPD)**

# Figure 2

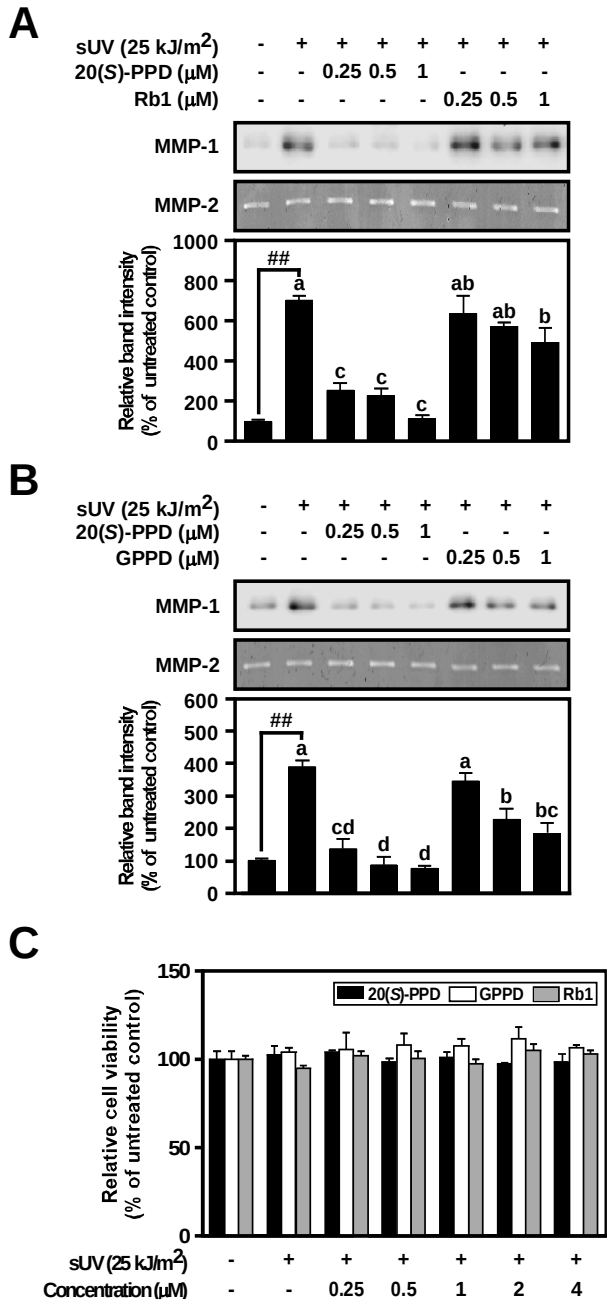
A
sUV (25 kJ/m2) - + + + + + + +
20(S)-PPD (μM) - - 0.25 0.5 1 - - -
Rb1 (μM) - - - - - 0.25 0.5 1
MMP-1
MMP-2
Relative band intensity (% of untreated control)
1000
800
600
400
200
0
##
a
c
c
c
ab
ab
b
B
sUV (25 kJ/m2) - + + + + + + +
20(S)-PPD (μM) - - 0.25 0.5 1 - - -
GPPD (μM) - - - - - 0.25 0.5 1
MMP-1
MMP-2
Relative band intensity (% of untreated control)
600
500
400
300
200
100
0
##
a
cd
d
d
a
b
bc
C
Relative cell viability (% of untreated control)
150
100
50
0
20(S)-PPD
GPPD
Rb1
sUV (25 kJ/m2) - + + + + + +
Concentration (μM) - - 0.25 0.5 1 2 4

**Figure 2. Effect of 20(*S*)-PPD on solar ultraviolet (UV)-induced matrix metalloproteinase (MMP)-1 protein expression in human keratinocytes (HaCaT).**

*A.* Effect of 20(*S*)-PPD on MMP-1 expression compared to ginsenoside Rb1. *B.* Effect of 20(*S*)-PPD on MMP-1 expression compared to GPPD. HaCaT cells were pretreated with 20(*S*)-PPD, Rb1, and GPPD at the indicated concentrations for 1 h, and then further treated with 25 kJ/m$^2$ solar UV for 48 h at 37°C. Protein expression was analyzed by Western blot assay. MMP-2 as loading control was determined by gelatin zymography. Data ($n$ = 3) represent the mean ± SEM. *C.* Cell viability of 20(S)-PPD, GPPD, and Rb1. The cell viability was measured by the CellTiter 96® AQueous One Solution Cell Proliferation Assay. Cells were pretreated with 20(*S*)-PPD, GPPD, and Rb1 at the indicated concentrations for 1 h, and then further treated with 25 kJ/m$^2$ solar UV for 48 h at 37°C. Data ($n$ = 3) represent the mean ±

SEM. Means with letters (a-c) in a graph are significantly different from each other at $p < 0.05$. $^{\#\#}p < 0.01$, relative to control cells.

### 3.2. 20(*S*)-PPD decreases solar UV-induced MMP-1 gene transcription and promotor activity resulting from suppression of AP-1 transactivation.

Pretreatment of 20(*S*)-PPD significantly suppressed solar UV- induced MMP-1 mRNA levels in HaCaT cells (Fig. 3A). To see the transcriptional regulation, the MMP-1 gene promotor activity was investigated and 20(*S*)-PPD decreased it in dose-dependent (Fig. 3B). Solar UV affected to stimulate the transcription factor AP-1, which regulate MMP-1 transcription [4]. I examined effect of 20(*S*)-PPD on AP-1 transactivation induced by solar UV irradiation in HaCaT cells in succession. 20(*S*)-PPD inhibited solar UV-induced AP-1 transactivation in HaCaT cells (Fig. 3C). These results indicate that 20(*S*)-PPD suppresses solar UV-induced MMP-1 gene transcription by suppressing AP-1 activity in HaCaT cells.

# Figure 3

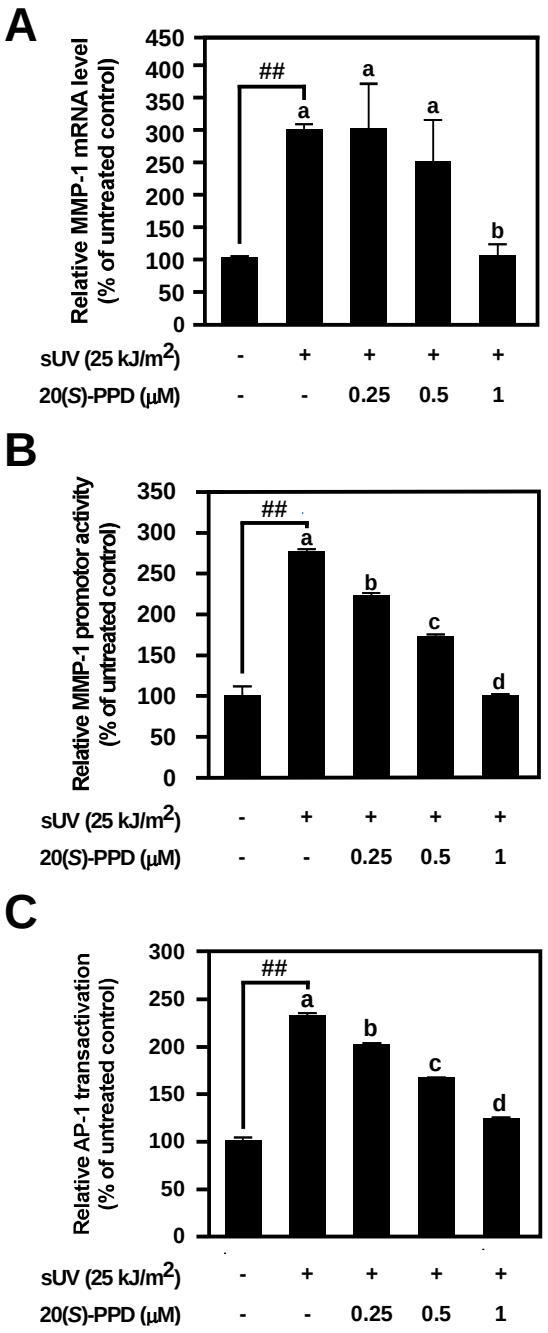
A
Relative MMP-1 mRNA level
(% of untreated control)
450
400
350
300
250
200
150
100
50
0
##
a
a
a
b
sUV (25 kJ/m2)
- + + + +
20(S)-PPD (μM)
- - 0.25 0.5 1
B
Relative MMP-1 promotor activity
(% of untreated control)
350
300
250
200
150
100
50
0
##
a
b
c
d
sUV (25 kJ/m2)
- + + + +
20(S)-PPD (μM)
- - 0.25 0.5 1
C
Relative AP-1 transactivation
(% of untreated control)
300
250
200
150
100
50
0
##
a
b
c
d
sUV (25 kJ/m2)
- + + + +
20(S)-PPD (μM)
- - 0.25 0.5 1

**Figure 3. Effect of 20(*S*)-PPD on solar UV-induced MMP-1 gene transcription, gene promotor activity, and activator protein (AP)-1 transactivation in HaCaT cells.**

*A.* MMP-1 mRNA level was determined by Real-time quantitative PCR. Cells were pretreated with 20(*S*)-PPD for 1 h, and then further treated with 25 kJ/m$^2$ solar UV for 24 h at 37°C. Data ($n$ = 3) represent the mean values ± SEM. *B.* Effect of 20(*S*)-PPD on MMP-1 promotor activity. HaCaT cells were pretreated with 20(*S*)-PPD at indicated concentrations for 1 h, and exposed 25 kJ/m$^2$ solar UV at 37°C. Cell extracts were collected after 8 h. MMP-1 promotor activity was measured using a luciferase reporter gene assay. Data ($n$ = 3) represent the mean values ± SEM. *C.* AP-1 transactivation ability of 20(*S*)-PPD. HaCaT cells were pretreated with 20(*S*)-PPD at the indicated concentrations for 1 h, and then further treated with 25 kJ/m$^2$. Cell extracts were collected after 4 h. AP-1 transactivation was measured

using a luciferase reporter gene assay. Data ($n$ = 3) represent the mean values ± SEM. Means with letters (a-d) in a graph are significantly different from each other at $p < 0.05$. $^{\#\#}p < 0.01$, relative to control cells.

### 3.3. 20(*S*)-PPD suppresses solar UV-induced mitogen-activated protein kinases (MAPKs) phosphorylation in HaCaT cells.

Next, I investigated the molecular mechanism of 20(*S*)-PPD in HaCaT cells. Based on the previous study, regulating MAPKs signaling pathway has a pivotal role in suppressing MMP-1 expression [4, 38]. Therefore, I examined the effect of 20(*S*)-PPD on solar UV-induced phosphorylation and total amount of these kinases in HaCaT cells. I verified that 20(*S*)-PPD suppressed solar UV-induced phosphorylation of MEK1,2/ERK/P90RSK and MEK3,6/P38 in a dose-dependent (Fig. 4A, B). In contrast, 20(*S*)-PPD did not reduce MKK4/JNK and Akt/$P70^{S6K}$ signaling pathways (Fig. 4C, D). As a result, these results indicate that 20(*S*)-PPD suppresses solar UV-induced MMP-1 gene transcription by suppressing AP-1 activity that was regulated by ERK- and p38-mediated signaling pathways.

# Figure 4

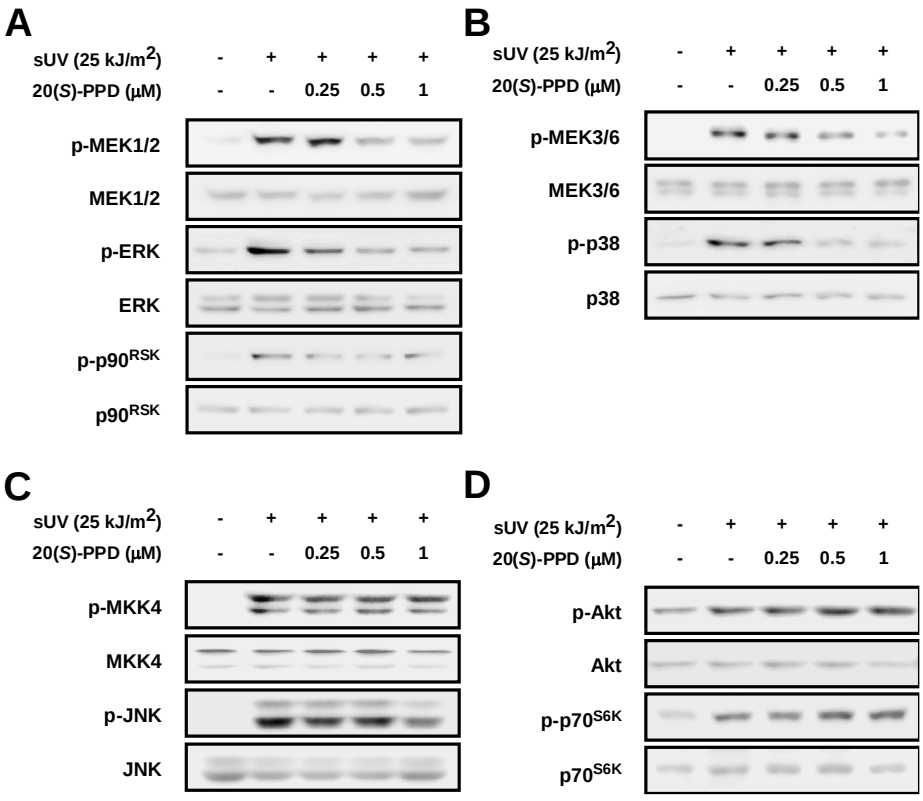
A
sUV (25 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
p-MEK1/2
MEK1/2
p-ERK
ERK
p-p90RSK
p90RSK
B
sUV (25 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
p-MEK3/6
MEK3/6
p-p38
p38
C
sUV (25 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
p-MKK4
MKK4
p-JNK
JNK
D
sUV (25 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
p-Akt
Akt
p-p70S6K
p70S6K

**Figure 4. Inhibitory effect of 20(*S*)-PPD on solar UV-induced signaling pathways.**

*A.* Effect of 20(*S*)-PPD in solar UV-induced phosphorylation of MEK1,2/ERK/P90$^{RSK}$ signaling pathway. *B.* Effect of 20(*S*)-PPD in solar UV-induced phosphorylation of MEK3,6/p38 signaling pathway. *C-D.* Effect of 20(*S*)-PPD in solar UV-induced phosphorylation of MKK4/JNK and Akt/P70$^{S6K}$ signaling pathway. After 20(*S*)-PPD pretreatment and solar UV irradiation, the cells were lysed as described in the Materials and Methods. Phosphorylated and total form of indicated proteins was determined by Western blot.

### 3.4. 20(*S*)-PPD prevents solar UV-induced MMP-1 expression and collagen degradation in human skin equivalent.

To verify whether the anti-wrinkle effect of 20(*S*)-PPD applied to human, I examined the effect of 20(*S*)-PPD on collagenase (MMP-1) and collagen fibers in human skin equivalent as described Fig 5A. After 2 days of solar UV irradiation, cell extracts were collected and paraffin-embedded 3D human skin tissues were sectioned. Paraffin embedded sections is treated by Masson's trichrome staining and immunohistochemistry. 20(*S*)-PPD inhibited solar UV-induced MMP-1 expression (Fig. 5B-C, E) and collagen degradation (Fig. 5D, F). Taken together, 20(*S*)-PPD shows anti-wrinkle effect in human skin equivalent by inhibiting MMP-1 expression and collagen degradation.

# Figure 5

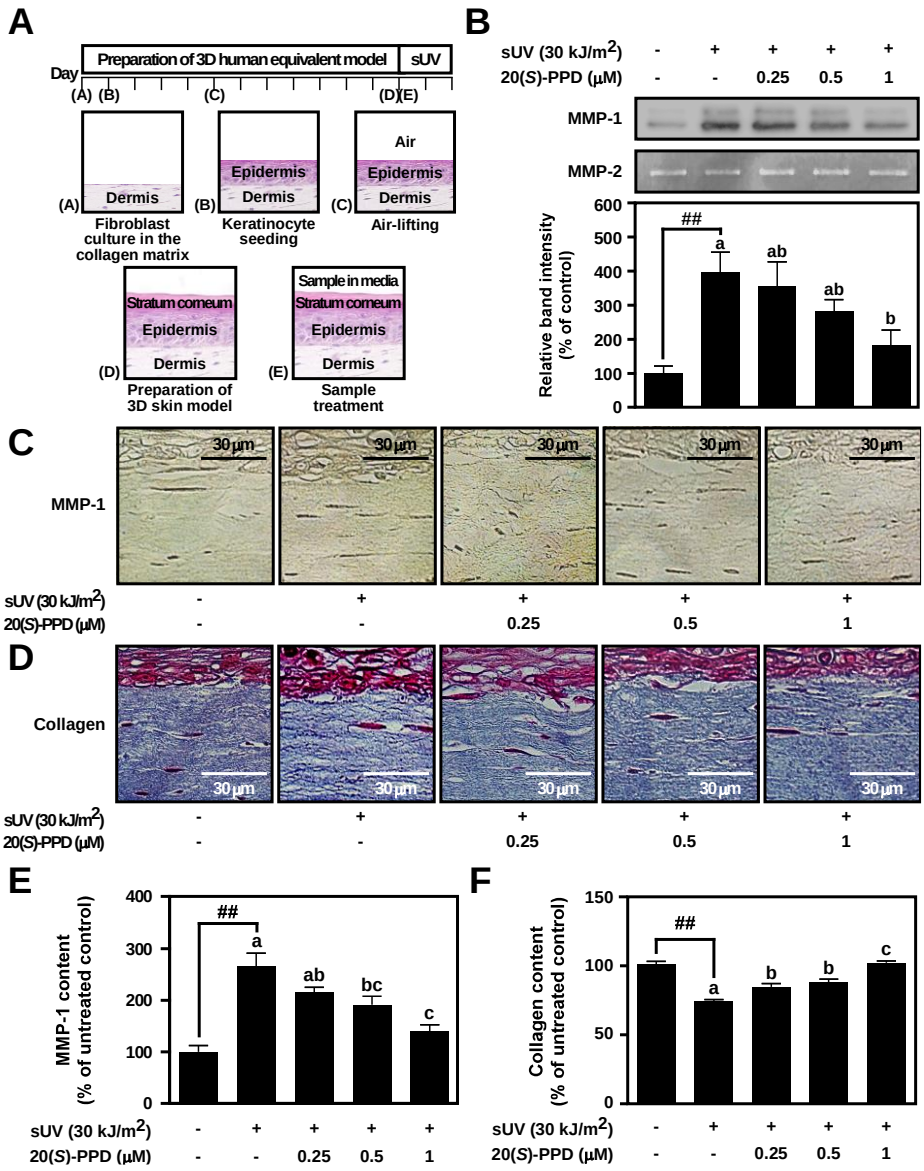
A
Preparation of 3D human equivalent model
sUV
Day
(A) (B)
(C)
(D)(E)
(A)
Dermis
Fibroblast culture in the collagen matrix
(B)
Epidermis
Dermis
Keratinocyte seeding
(C)
Air
Epidermis
Dermis
Air-lifting
Stratum corneum
Epidermis
Dermis
(D)
Preparation of 3D skin model
Sample in media
Stratum corneum
Epidermis
Dermis
(E)
Sample treatment
B
sUV (30 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
MMP-1
MMP-2
Relative band intensity (% of control)
600
500
400
300
200
100
0
##
a
ab
ab
b
C
MMP-1
30 μm
sUV (30 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
D
Collagen
30 μm
sUV (30 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
E
MMP-1 content (% of untreated control)
400
300
200
100
0
##
a
ab
bc
c
sUV (30 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1
F
Collagen content (% of untreated control)
150
100
50
0
##
a
b
b
c
sUV (30 kJ/m2) - + + + +
20(S)-PPD (μM) - - 0.25 0.5 1

**Figure 5. 20(*S*)-PPD inhibits solar UV-induced collagen degradation and MMP-1 expression in human skin equivalent.**

*A.* A schematic diagram of experiment. *B.* Effect of 20(*S*)-PPD on MMP-1 expression Protein expression was determined by western blot. *C.* Immunohistochemistry for visualizing MMP-1 level. MMP-1 appears dark brown color. *D*. Masson's trichrome staining for visualizing collagen fibers. Collagen fibers appear blue color. *E, F.* MMP-1 and collagen content data were quantified using the image J software analysis (Open source JAVA-written program, NIH, USA). Data ($n$ = 7) represent the mean values ± SEM. Means with letters (a-c) in a graph are significantly different from each other at $p < 0.05$. $^{\#\#}p < 0.01$, relative to control cells.

# Figure 6

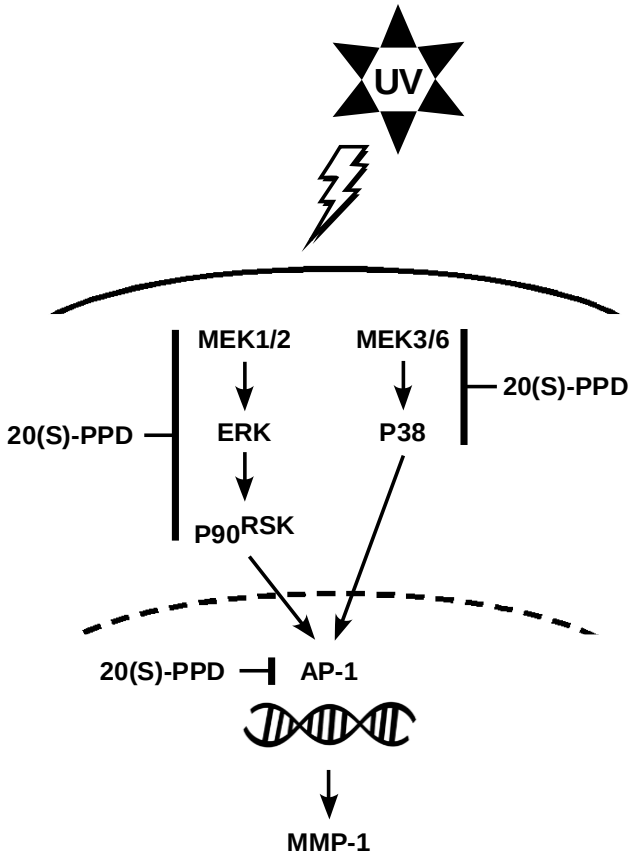


**Figure 6. Proposed mechanism of 20(*S*)-PPD**

20(*S*)-PPD inhibits on solar UV-induced MMP-1 expression by regulating MEK1,2/ERK/$P90^{RSK}$ and MEK3,6/p38 pathways in HaCaT cells.

# Ⅳ. DISCUSSION

Sun exposure is a major factor of premature skin aging process, and this phenomenon is called photoaging [15, 39]. Skin photoaging is a process of senescence and is commonly related to representative symptoms such as wrinkle, sagging, and laxity [40]. Solar UV irradiation results in the overexpression of MMP-1 which plays a major role in wrinkling by photoaging process [11].

Both UVA and UVB have an important effect on skin wrinkle by photoaging. It is important to use solar UV wavelengths with UVA and UVB to understand simultaneous biological processes of photoaging.

For validating the anti-wrinkle effect on skin, human skin equivalent and in vitro model were needed to make the results more reliable. Human skin equivalent mimics the morphology and physiology

of human. This human skin equivalent is often used to research diseases such as skin photoaging [41], inflammation [42] and ichthyosis [43]. Therefore, in order to test at the similar environment of actual skin, I used this method for measuring the anti-wrinkle effect of 20(*S*)-PPD.

Ginseng is widely used as the most traditional medicinal plants particularly in Korea, Japan and China [17]. There are a lot of studies that ginseng can help to prevent skin aging caused by UV irradiation [22, 23]. Moreover, both Rb1 and GPPD have been reported as the preventing effects on skin wrinkle, however anti-wrinkle effects about 20(*S*)-PPD, an aglycone derivative of Rb1 metabolite have not been reported yet.

Based on clinical experience, compounds with molecular weight (MW) of less than 500 Dalton are considered important factor of skin absorption. [44, 45]. Because the MW of 20(*S*)-PPD is approximatively 460 g/mol, it seems to pass the corneal layer compared

to other ginsenosides. It indicated that 20(*S*)-PPD can be a therapeutic agent for skin topical delivery. Therefore, I focused on the anti-wrinkle effects of 20(*S*)-PPD, and then monitored changes induced by solar UV.

In this study, I showed that 20(*S*)-PPD has anti-wrinkle effect in human skin equivalent model through evaluation of staining of specific protein, collagen and MMP-1 used as major biomarker in skin wrinkle research. 20(*S*)-PPD inhibited MMP-1 expression and collagen degradation in human skin equivalent. In addition, I revealed that 20(*S*)-PPD has anti-wrinkle effect by suppressing solar UV-induced MMP-1 protein expression and MMP-1 gene transcription in HaCaT cells compared to Rb1 and GPPD. And these regulations of MMP-1 are derived from reduction of UV-induced AP-1, which is the major transcription factor of MMP-1 activity. Therefore, 20(*S*)-PPD has been suggested to be a beneficial agent for the treatment of solar UV-induced wrinkle.

Upon UV irradiation, MAPKs signal transduction pathway is activated, which mediates downstream cellular response [46, 47]. Phosphorylation of MAPKs, which consists of ERK, JNK, and p38 promotes activation of AP-1 [48]. By downregulating phosphorylation of MEK1,2/ERK/P90$^{RSK}$ and MEK3,6/p38 pathways, 20(*S*)-PPD inhibits activity of AP-1 which result in upregulation of MMP-1 that contributed to collagen degradation.

In conclusion, these results indicate that 20(*S*)-PPD protect against solar UV-induced skin wrinkle in human skin equivalent and HaCaT cells. The role of 20(*S*)-PPD in wrinkle formation has been discovered through regulating MMP-1 transcription which involves decreased AP-1 activity by suppression of MEK1,2/ERK/P90$^{RSK}$ and MEK3,6/p38 pathways. Also I find out that an anti-wrinkle effect of 20(*S*)-PPD is superior to its precursors, Rb1 and GPPD. Therefore,

20(*S*)-PPD could be used as a beneficial agent to be used as therapeutic anti-wrinkle agents.

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# Ⅵ. 국문 초록

인삼은 한국, 중국을 비롯한 여러 아시아국가에서 다양한 질병을 치료하기 위한 전통 약재로 사용되어 왔으며, 인삼의 약리활성의 주요성분은 진세노사이드로 알려져 있다.

자외선(UV)에 노출된 피부는 콜라겐분해효소(MMP-1)에 의해 주름 형성과 탄력 저하가 가속화된다. 인삼이 UV에 의해 유도되는 광노화에 의한 피부 주름을 억제한다는 연구 결과는 다수 보고되어 있으나, 인삼 대사체인 20(*S*)-PPD의 피부 주름 개선 효능은 보고된 바 없다. 본 연구는 진세노사이드 대사체인 20(*S*)-PPD의 자외선에 의한 피부 주름 개선 효능에 대해 규명하였다.

UVA와 UVB를 태양광과 유사하도록 조합한 파장을 조사하여 유도되는 MMP-1 발현 억제 효능을 조사한 결과 20(*S*)-PPD는 농도 의존적으로 MMP-1 발현을 감소시켰다. 또한, Rb1과 프

로토파낙사다이올계 진세노사이드 중 가장 우수한 MMP-1 발현 억제 효능을 보인다고 알려진 GPPD보다 우수한 저해 효능을 보인다. 20(*S*)-PPD은 MEK1,2/ERK/P90$^{RSK}$ 및 MEK3,6/p38의 신호전달체계 단백질의 인산화를 억제함으로써 MMP-1의 주요 전사 인자인 AP-1의 활성을 저해한다. 이를 통해 MMP-1의 전사 과정이 저해되면서 발현이 감소한다. 생체피부모방모델에서 수행된 실험에서도 20(*S*)-PPD에 의해 콜라겐 분해 정도 및 MMP-1 발현이 유의적으로 감소했다.

본 연구를 통해 20(*S*)-PPD는 위에 제시한 하위 신호전달과정의 조절을 통해 MMP-1의 전사 과정을 저해하여 피부 주름을 발생시키는 MMP-1 발현을 억제함을 규명하였다. 이를 바탕으로 자외선에 의한 피부 주름을 개선하는 신규 천연물로서의 20(*S*)-PPD의 가능성을 제시하였다.