A Thesis for the Degree of Master of Science

Orobol prevents solar ultraviolet-induced skin photoaging by targeting T-LAK Cell-originated Protein Kinase

오로볼의 자외선에 의한 피부 주름 개선 효능 및 작용기작 규명

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ABSTRACT

Soy isoflavones are attractive source of functional cosmetic and food materials with anti-wrinkle, whitening and skin hydration effects. After fermentation or bioconversion, the majority of soy isoflavones are transformed to their metabolites. Recently, metabolites have been studied in their improved bioactivity. Matrix metalloproteinase (MMP)-1 play a critical role in the degradation of collagen in skin, thereby accelerating the photo-aging process of skin. In this study, I investigated the anti-photoaging effect of orobol, a major metabolite of genistein in soybean. Orobol reduced sUV-induced MMP-1 expression in human keratinocyte (HaCaT) cells and human dermal fibroblasts (HDFs). It further inhibited MMP-1 mRNA transcription. And the transactivation of AP-1, a major transcription factor of MMP-1, is also suppressed. From a 359 whole human kinase profiling assay, T-LAK Cell-originated Protein Kinase (TOPK) was suggested as a direct target of orobol. Additionally, orobol decreased sUV induced phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinases (JNK) and p38 mitogen-activated protein kinase (p38) which are downstream of TOPK. In addition, pull-down assay showed that orobol directly bound TOPK in ATP non-competitive manner. Overall, orobol produced the anti-photoaging effect

by directly targeting TOPK and suppressing sUV-induced MMP-1 expression in HaCaT cells and HDFs. Thus, orobol could be a beneficial agent for reducing sUV-induced wrinkle formation.

Keywords: Orobol; Kinase profiling; MMP-1; TOPK; solar UV; photoaging

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CONTENTS

Ⅰ. INTRODUCTION

Skin is the largest organ of the human body which has importance functions as physical and chemical barrier [1]. Moreover, skin anti-aging is considered an important priority in the overall beauty care, according to the demand for ways to look younger. The prevention of skin aging is necessary for maintaining a younger-looking appearance [2]. Skin aging is divided intrinsic aging which result from the passage of time and extrinsic aging caused by external influences [3]. Intrinsically aged skin has slow and partly irreversible degeneration of connective tissue, related to passage of time and genetic factors. Extrinsically aged skin is caused by environmental factors such as solar ultraviolet, air pollution, and high temperature. It consists rapidly thickened, tangled, and ultimately regular amorphous elastic structures [4, 5].

Among the environmental factors, solar UV (sUV) is the major factor of fastening skin aging and called photoaging [6]. Sun-exposed skin features deep wrinkles, dryness, uneven pigmentation, and an accelerated decline in the function of skin [7]. sUV-damaged skin cells produce abnormal elastin resulting from chronic enzymatic digestion of extracellular matrix [4]. Collagen degradation is the prominent feature of photoaging that follows chronic exposure of sUV radiation. After sUV irradiation, endogenous photosensitizer molecules absorb the energy, following generation of reactive

oxygen species (ROS) which brings severe oxidative stress in skin [8]. ROS activates cytoplasmic signal transduction pathways in resident skin cells that are related to growth and differentiation. It induces various inflammatory signaling pathway and alters signaling cascades [9]. As a result, sUV irradiation rapidly enhances activation of transcription factor AP-1 [7,10]. The AP-1 subsequently upregulates matrix-metalloproteinase (MMP) expression strongly [7]. In various MMPs, MMP-1 is known as interstitial collagenase [11-13]. Its expression is associated with connective tissue remodeling such as wound healing, tissue morphogenesis, angiogenesis and metastasis [13]. Especially, type Ⅰ and type Ⅲ collagens are the most abundant proteins in the skin layers, consisting extended mechanically stiff fibrils and tensile strength to the tissue [3]. MMP-1 specifically collapses the type Ⅰ and type Ⅲ collagens which are consisted of extracellular matrix (ECM) [14]. Overexpressing MMP-1 decreases procollagen production and breaking down collagen [6, 10, 15]. Therefore, inhibition of MMP-1 expression induced by sUV irradiation is one of the excellent strategies for preventing skin damage and photoaging.

T-LAK Cell-originated Protein Kinase (TOPK) is a protein kinase related MAP kinase kinase (MAPKK) family. TOPK is expressed in variety of cells such as activated T-LAK cells, lymphoma, myeloma, leukemia and

colorectal cancer (CRC) cells [16]. It acts oncogenic function in colon cancer and breast cancer cells [17, 18]. TOPK is an upstream activator of MAPKs; extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinases (JNK) and p38 MAPK (p38). And it was reported that phosphorylation level of MAPKs is regulated after transfection of TOPK [17, 19-21]. Besides, TOPK is phosphorylated by sUV irradiation and it affects the activity of transcription factor AP-1 [22]. However, it is still unclear for the role of TOPK in MMP-1 expression.

Soybeans (*Glycine max*) are typical food for skin health from natural sources [23]. The effect is dependent on the active ingredient isoflavone in soybean [24]. In particular, genistein (4',5,7-trihydroxyisoflavone) is the most abundant isoflavone in a soybean [25]. It has been shown health beneficial effects like cancer, cardiovascular disease and its phytoestrogen activity [26]. The studies of its helpful effects in skin are also reported including skin moisturing via stimulating hyaluronic acid in HaCaT cells, inhibiting oxidation of skin, and preventing skin wrinkle formation and cancer [27, 28]. As well, soybean isoflavones have effects on synthesis of type Ⅰ collagen and cell proliferation [29]. Orobol (7,8,3',4'-tetrahydroxyisoflavone) is a metabolite of genistein produced in human liver microsomes after soy consumption [30]. Orobol occurred in fermented soybean is produced from *Tritirachium* or

Aspergillus niger [31]. Furthermore, It could be produced by hydroxylation of genistein with tyrosinase from *Bacillus megaterium* [32]. Several studies have shown that orobol has anticarcinogenic effects against ovarian and breast cancer cell lines [33, 34]. However, the effect of orobol for skin phogoating and its molecular target has not been studied yet.

In this study, I showed orobol has most inhibitory effect of sUV-induced MMP-1 expression among various isoflavones in HaCaT cells and human dermal fibroblasts (HDFs). As well, orobol suppressed sUV-induced MMP-1 transcription and AP-1 transactivation. Furthermore, after kinase profiling, I suggested TOPK as a direct target of orobol and tried to prove it.

Ⅱ. MATERIALS AND METHODS

2.1. Chemicals and reagents

Orobol is gently provided for Prof. Byung-Gee Kim laboratory (Seoul National University, Seoul, Korea). Orobol was o-hydroxylated by genistein with > 95% conversion yield. It was biotransformed by tyrosinase expressed from *Bacillus megaterium* [32]. Genistein, genistin, daidzein, daidzin and equol were purchased from Sigma-Aldrich (St. Louis, MO). 6,7,4'-Trihydroxyisoflavone (6-ODI) was obtained from Chromadex™ (Irvine, CA). 7,3'4'-Trihydroxyisoflavone (3'-ODI) and 7,8,4'-Trihydroxyisoflavone (8-ODI) were purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). Dulbecco's modified eagle medium (DMEM) was purchased from Hycolne (Long, UT). Fetal bovine serum (FBS) was bought from Sigma-Aldrich. Penicillin-Streptomycin Solution was purchased from Mediatech, Inc. (Manassas, VA). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was purchased from Promega Corporation (Madison, WI). The MMP-1 antibody was obtained from R&D systems Inc. (Minneapolis, MN). Antibodies against phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, total JNK1/2, phosphorylated p38 (Thr180/Tyr182), and total p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other

antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA). The Protein assays reagent kits were obtained from Bio-Rad Laboratories (Hercules, CA).

2.2. Cell culture and treatments

HDFs were isolated from the outgrowth of foreskin obtained 14 year-olds healthy volunteer from Dr. Chung JH laboratory (Seoul National University Hospital, Seoul, Korea) under the Institutional Review Board at Seoul National University Hospital (No. H-1101-116-353) and Seoul National University (No. E1511/001-011). HaCaT cells were purchased form CLS (CLS Cell Lines Services GmbH, Heidelberg, Germany). Both cells were cultured in DMEM with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin at 37 °C and 5 % CO_2.

2.3. sUV irradiation

sUV 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 by a UV meter and was 94.5 % and 5.5 % respectively. HDFs were exposed to sUV at a dose of 35

kJ/m^2, and HaCaT cells were exposed to sUV at 25 kJ/m^2 in serum free media.

2.4. Cell viability

The cell cytotoxicity was measured using the MTS assay. HDFs were cultured in the 96 well plates at a density of 2×10^4 cells/well and incubated in DMEM-10% FBS containing penicillin/streptomycin at 37 °C in a 5 % CO_2 atmosphere, and HaCaT cells were cultured in the 96 well plates at a density of 10×10^4 cells/well and incubated at same condition with HDFs. Cells were starved in serum-free DMEM for 24 h. The cells and each samples were incubated for 48 h at 37 °C after sUV irradiation, and treated with MTS solution which activated by PMS solution for 2 h. The absorbance at 490 nm was measured by using a microplate reader (Molecular Devices, CA).

2.5. Western blot

HDFs and HaCaT cells were cultured for 48 h and the cells were incubated in serum-free DMEM for 24 h. After that, the cells were treated with or without various concentrations of orobol, genistein (1, 2 and 4 μM) and the other isoflavones (4 μM) for 1 h, followed by sUV (35 kJ/m^2 for HDFs, 25 kJ/m^2 for HaCaT cells) 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, Darmstadt, Germany). The membrane was blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, Little Chalfont, UK) after hybridization with the HRP-conjugated secondary antibody (Life technologies. Waltham, MA). Western blot data were quantified using the program ImageJ (National Institutes of Health, Bethesda, MD).

2.6. Gelatin zymography

Zymography was performed in 12 % polyacrylamide gel with the presence of gelatin (0.1 % w/v) as a substrate for MMP-2. The protein samples were mixed with loading buffers [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 renaturating buffer (Life technologies) for 1 h at room temperature and incubated for 16 h at 37 °C in developing buffer (Life technologies). After enzyme reaction, the gel was stained with 0.5 % Coomassie brilliant blue in 10 % acetic acid.

2.7. Real-time quantitative PCR

HDFs were treated with orobol and genistein for 32 h and HaCaT cells for 24 h. Samples were harvested in RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After reverse transcript with oligo-dT primers using a PrimeScript^{TM} 1st 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 °C for 3 min. Amplification was made up of 44 cycles at 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 30. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). 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').

2.8. 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-MMP-1-mCMV-EF1-puro vector was gently provided by Dr, Sung-Keun Jung (Korea Food Research Institute, Sung-Nam, Korea). MMP-1 promoter is coloned to pGF vector [13]. pGF-MMP-1-mCMV-EF1-puro vectors and pGF-AP1-mCMV-EF1-Puro vectors 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 into 24 h after transfection and the cells were then cultured for 36 h. The viral particles were harvested by filtration using a 0.45 mm syringe filter, then combined with 8 μg/mL polybrenes (MERK Millipore) and infected into 60 % confluent HDFs and HaCaT cells overnight. The cell cultured medium was replaced with fresh complete growth medium for 24 h before the cells were selected for using puromycin (Sigma-Aldrich) over 36 h. The selected cells were then used for further experiments. The selected HaCaT cells and HDFs were cultured for 48 h and then starved to serum-free DMEM for 24 h. After starvation, HDFs were treated with or without various concentrations of orobol and genistein for 1 h, followed by 35 kJ/m^2 sUV irradiation, and HaCaT cells by 25 kJ/m^2. Cell extracts were prepared for reporter lysis buffer (Promega). HaCaT cells for MMP-1 were lysated after 12 h and HDFs after 24 h. And HaCaT cells for AP-

1 were lysated after 6 h and HDFs after 12 h. The extracts were used for luciferase assay. MMP-1 and AP-1 activity in HDFs and HaCaT cells were determined by using an luciferase assay kit (Promega), as described by the manufacturers.

2.9. *in vitro* and *ex vivo* pull down assay

Sepharose 4B freeze-dried powder (0.3 g; GE Healthcare) was activated in 1 mM HCl and suspended in orobol (2 mg) coupled solution (0.1 M NaHCO3 and 0.5 M NaCl). Following overnight rotation at 4°C, the mixture was transferred to 0.1 M Tris-HCl buffer (pH 8.0) and again further rotated at 4°C overnight. The mixture was washed three times with 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris-HCl + 0.5 M NaCl buffer (pH 8.0), respectively, and suspended in PBS. The pull down assay was performed as previously described. In brief, active protein (TOPK) was incubated with Sepharose 4B alone or orobol-Sepharose 4B beads in reaction buffer. After incubation at 4°C, the beads were washed in washing buffer and proteins bound to the beads were analyzed by immunoblotting.

2.10. Kinase profiling and kinase assay

Kinase profiling and kinase assay were conducted by kinase assay services (Reaction Biology Corporation, Malvern, PA). Kinases are incubated

with substrate and required cofactor. The reaction is initiated by the addition of the compound in dimethyl sulfoxide (DMSO) and ^{33}P-ATP (specific activity 10 μCi/μl). After incubation for 120 min at room temperature, reaction is spotted onto P81 ion exchange paper (GE healthcare) and washed extensively in 0.75 % phosphoric acid. Kinase activity data was expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions.

2.11. Statistical analysis.

Results are presented as means ± standard deviation (SD). Differences between control and sUV-irradiated control were assessed with Student's *t*-test. To compare the difference between the sUV-exposed groups, one-way ANOVA was used with Duncan. The data were statistically analyzed with IBM SPSS Statistics ver. 22.0 (IBM Co., Armonk, NY) and P values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

3.1. Orobol has inhibitory effect on sUV-induced MMP-1 protein expression in HaCaT cells and HDFs.

The structure and bioconversion flow of genistein and orobol showed in Figure 1A. To investigate the most effective isoflavones for repressing s UV-induced MMP-1 expression, the effect of 9 abundant isoflavones including orobol were compared with orobol in HaCaT cells and HDFs. Genistein, genistin, daidzein, daidzin, 6-ODI , 3'-ODI, 8-ODI and equol were used as a compared isoflavones. As a result, orobol showed the greatest inhibitory effect among the isoflavones (Fig. 1B). Also orobol decreased MMP-1 protein expression of a concentration-dependent manner on HaCaT cells (Fig. 1C) and HDFs (Fig. 1D). The cell viability of orobol and genistein was evaluated using MTS assay on HaCaT cells (Fig. 1E) and HDFs (Fig. 1F). In both cells, orobol did not represent cytotoxicity within 4 μM in the presence of sUV irradiation. (Fig. 1E and 1F).

Figure 1

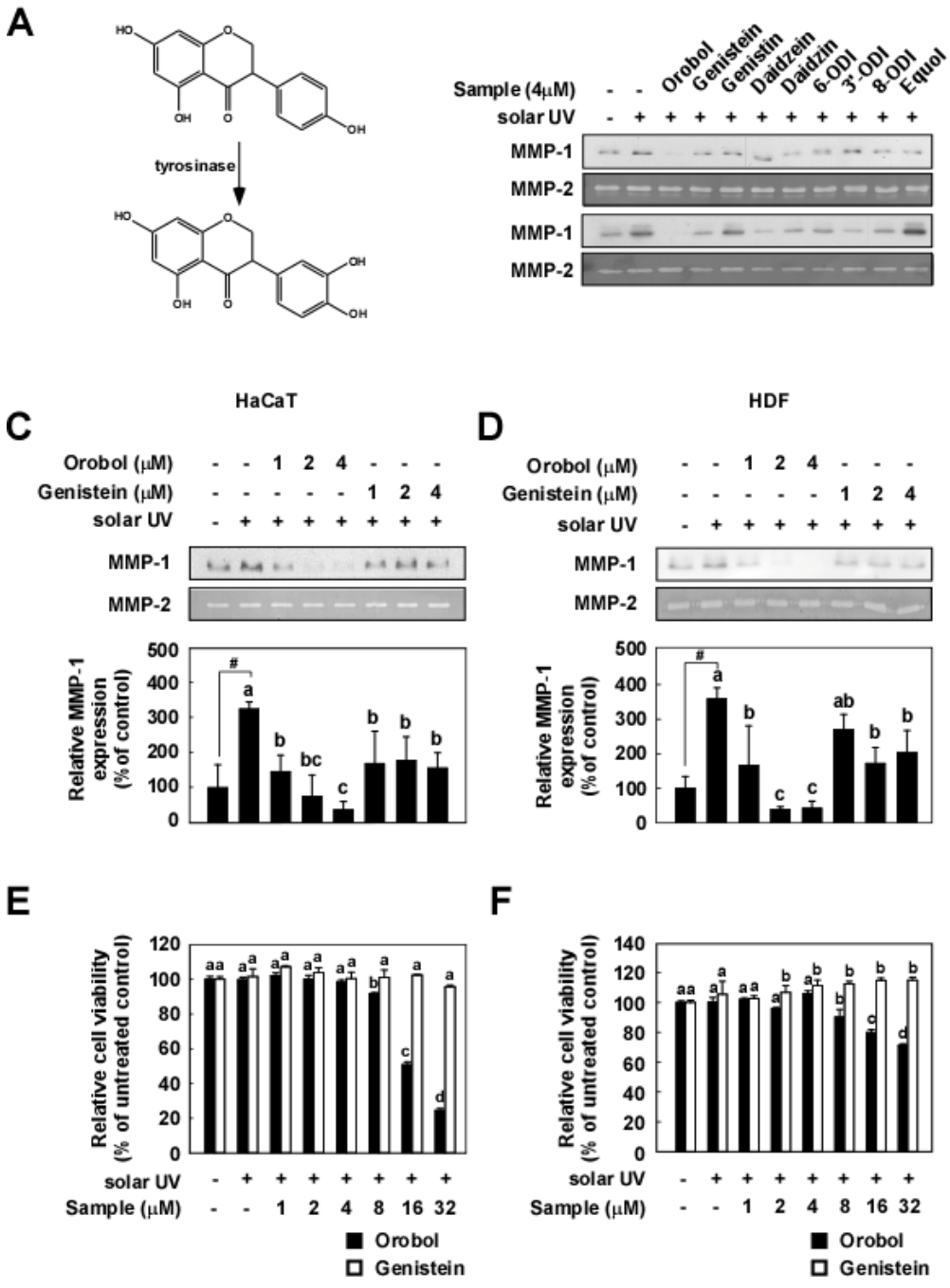
A
tyrosinase
Sample (4μM)
Orobol
Genistein
Genistin
Daidzein
Daidzin
6-ODI
3'-ODI
8-ODI
Equol
solar UV
MMP-1
MMP-2
C
HaCaT
Orobol (μM)
Genistein (μM)
Relative MMP-1 expression (% of control)
D
HDF
E
F
Relative cell viability (% of untreated control)
Sample (μM)
Orobol
Genistein

Figure 1. Orobol shows the greatest effect of sUV-induced MMP-1 activity among the isoflavones.

A. Chemical structures of orobol and genistein. **B.** Effect of orobol on MMP-1 expression compared to various isoflavones. Isoflavones (4 μM) were added to human keratinocyte (HaCaT) cells and human dermal fibroblasts (HDFs) for 1 h before being exposed to 25 kJ/m^2 sUV on HaCaT cells and 35 kJ/m^2 on HDFs. After 48 h of solar UV (sUV) irradiation, protein expression was analyzed by western blotting (MMP-1) and zymography (MMP-2). Upper two lines are results of expression in HaCaT cells and the other two lines are results in HDFs. **C and D.** Effect of orobol on MMP-1 expression was compared to genistein. MMP-1 was determined by western blot. MMP-2 was used as a loading control by zymography. Both cells were pretreated with orobol and genistein at the indicated concentrations for 1 h, and then exposed to 25 kJ/m^2 sUV on HaCaT cells (*C*) and 35 kJ/m^2 on HDFs (*D*). MMP-1 protein expression data was quantified using the image J software (NIH). Data (n=3) represents the mean values ± SD. **E and F.** Cell viability of orobol and genistein. Both cells were respectively pretreated with orobol and genistein at the indicated concentrations for 1 h, and exposed 25 kJ/m^2 sUV on HaCaT cells (*C*) and 35 kJ/m^2 on HDFs (*D*) at 37 ℃. After 48 h,

cell viability was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay as described in the material and methods. Data (n=5) were shown as the means ± SD.

3.2. Orobol decreases sUV-induced MMP-1 mRNA transcription and AP-1 transactivation.

To understand whether the anti-photoaging effects of orobol is in gene expression level, mRNA expression and transactivation of MMP-1 was investigated. Orobol decreased MMP-1 mRNA level in HaCaT cells and HDFs (Fig. 3A and 3B). And the level of MMP-1 transactivation was decreased it in dose-dependent manner (Fig 3C and 3D). Since the sUV-induced transcription of MMP-1 is dependent on AP-1 transcription factor [7, 15], I examined levels of AP-1 transactivation in the presence of orobol in HaCaT cell and HDFs. sUV-induced AP-1 transactivity was inspected in the presence of orobol and genistein in HaCaT cells (Fig. 3E) and HDFs (Fig. 3F). Orobol inhibited AP-1 transactivity effectively and it is more than genistein. All these results were compared in identical doses with genistein.

Figure 2

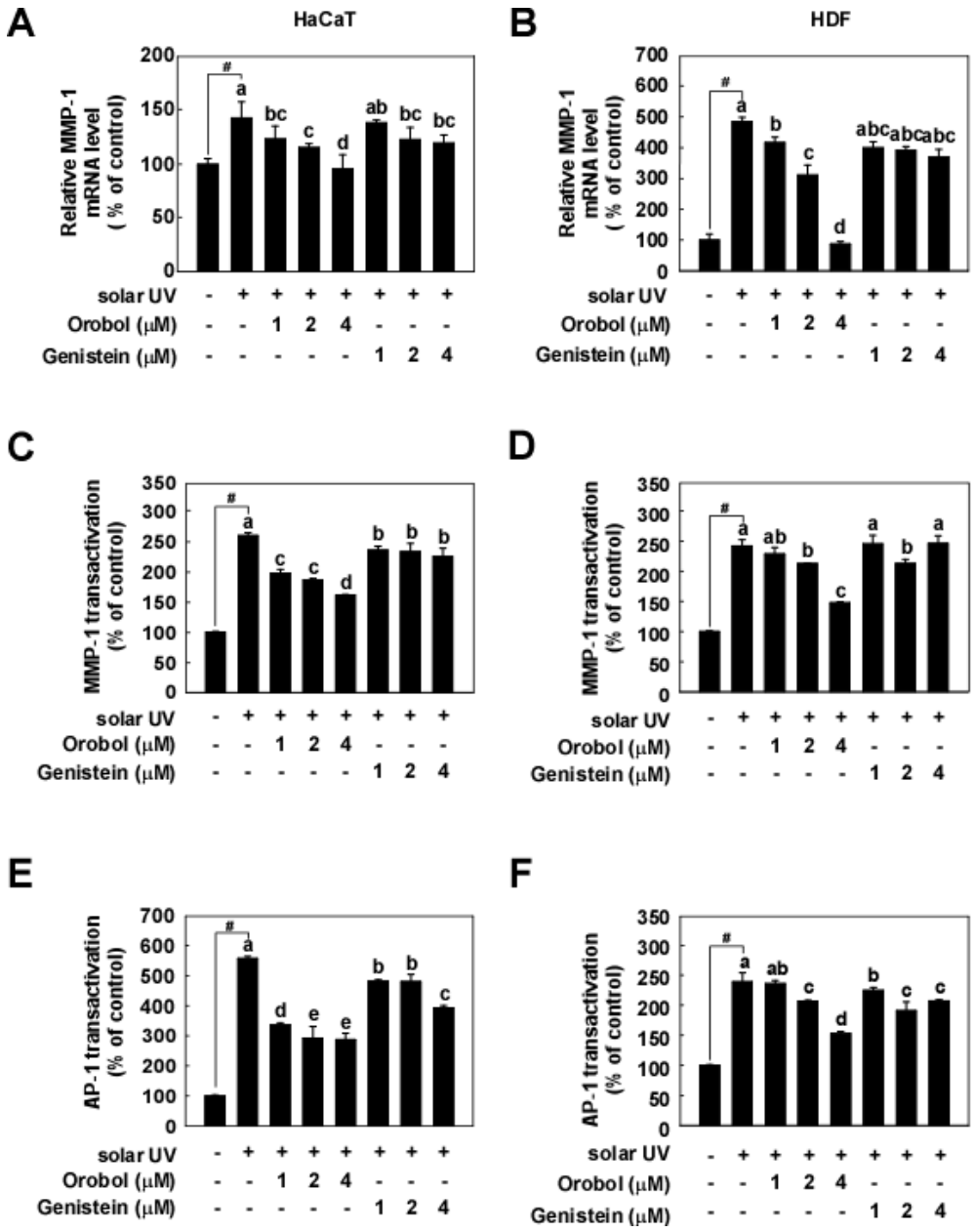
A
HaCaT
Relative MMP-1 mRNA level (% of control)
B
HDF
Relative MMP-1 mRNA level (% of control)
C
MMP-1 transactivation (% of control)
D
MMP-1 transactivation (% of control)
E
AP-1 transactivation (% of control)
F
AP-1 transactivation (% of control)
solar UV
Orobol (μM)
Genistein (μM)

Figure 2. Effect of orobol on sUV-induced MMP-1 transcription and AP-1 transactivation in HaCaT cells and HDFs.

A and B. MMP-1 mRNA levels of orobol and genistein. Both cells were pretreated with orobol and genistein at the indicated concentrations for 1 h, and exposed 25 kJ/m^2 sUV on HaCaT cells (*A*) and 35 kJ/m^2 on HDFs (*B*) 37 ℃. The mRNA was collected after 24 h in HaCaT cells (*A*), and 32 h in HDFs (*B*). It was analyzed as real-time quantitative PCR described in the Materials and Methods. Data (*n*=3) represented the mean values ± SD.

C and D. Effect of orobol and genistein on MMP-1 transactivation. Both cells were pretreated with orobol and genistein at the indicated concentrations for 1 h, and exposed 25 kJ/m^2 sUV on HaCaT cells (*C*) and 35 kJ/m^2 on HDFs (*D*) at 37 ℃. Cell extracts were collected after 10h in HaCaT cells (*C*) and 24 h in HDFs (*D*). MMP-1 transactivation was measured using a luciferase reporter gene assay as described in the Materials and Methods. Data (*n*=3) represented the mean values ± SD.

E and F. Effect of orobol and genistein on AP-1 transactivation. Both cells were pretreated with orobol and genistein at the indicated concentrations for 1 h, and exposed 25 kJ/m^2 sUV on HaCaT cells (*E*)

and 35 kJ/m^2 on HDFs (*F*) at 37 ℃. Cell extracts were collected after 6 h in HaCaT cells (*E*) and 24 h in HDFs (*F*). AP-1 transactivation was measured using a luciferase reporter gene assay as described in the Materials and Methods. Data (*n*=3) represents the mean values ± SD.

3.3. Orobol reduces the kinases activity including TOPK.

Kinase profiling analysis was charged to find a molecular mechanism of orobol in early time of sUV signaling pathway. First, 395 kinases activities were analyzed with 20 μM orobol (Table 1). Based on Table 1, 26 kinases inhibited more than 100% were selected. Kinase profiling analysis of selected kinases was conducted again with 1 μM of orobol (Table 2). Based on the results from Table 2, I choose MUSK, TNIK, MNK1, KHS/MAP4K5, CK1epsilon and TOPK, which were inhibited more than 75 % by orobol. First of kinases, I found kinase which was expressed in human skin from atlas database [35], activated in sUV irradiation and affected AP-1 activation [36]. As a result, I suggested TOPK as a main target of orobol.

Table 1. Kinase screening of orobol (20 μM)

Kinase profiling analysis was performed on whole human kinases (359 kinases) to find the target of orobol (20 μM) by kinase assay service (Reaction Biology Corporation). Data are representative of two independent experiments that give similar results.

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
ABL1	17	ALK2 /ACVR1	64	Aurora A	4	BRK	39	CAMK1a	-5
ABL2/ARG	18	ALK3 /BMPR1A	17	Aurora B	2	BRSK1	1	CAMK1b	0
ACK1	-2	ALK4 /ACVR1B	3	Aurora C	5	BRSK2	2	CAMK1d	-1
AKT1	30	ALK5 /TGFBR1	0	AXL	4	BTK	-2	CAMK1g	2
AKT2	28	ALK6 /BMPR1B	35	BLK	2	c-Kit	4	CAMK2a	1
AKT3	12	ARAF	6	BMPR2	1	c-MER	3	CAMK2b	0
ALK	0	ARK5 /NUAK1	7	BMX /ETK	1	c-MET	0	CAMK2d	2
ALK1 /ACVRL1	15	ASK1 /MAP3K5	2	BRAF	11	c-Src	1	CAMK2g	1

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
CAMK4	2	CDK2 /cyclin A	6	CDK7 /cyclin H	62	CK1g3	0	DAPK2	1
CAMKK1	9	CDK2 /Cyclin A1	38	CDK9 /cyclin K	12	CK2a	2	DCAMKL1	39
CAMKK2	6	CDK2 /cyclin E	10	CDK9 /cyclin T1	4	CK2a2	0	DCAMKL2	58
CDC7 /DBF4	8	CDK2 /cyclin O	5	CHK1	66	CLK1	0	DDR1	6
CDK1 /cyclin A	16	CDK3 /cyclin E	15	CHK2	6	CLK2	1	DDR2	5
CDK1 /cyclin B	10	CDK4 /cyclin D1	10	CK1a1	4	CLK3	3	DLK /MAP3K12	2
CDK1 /cyclin E	25	CDK4 /cyclin D3	11	CK1a1L	7	CLK4	1	DMPK	127
CDK14 /cyclin Y	46	CDK5/p25	3	CK1d	3	COT1/MAP3K8	16	DMPK2	2
CDK16 /cyclin Y	24	CDK5/p35	7	CK1epsilon	-1	CSK	3	DRAK1 /STK17A	6
CDK17 /cyclin Y	49	CDK6 /cyclin D1	23	CK1g1	0	CTK /MATK	28	DYRK1 /DYRK1A	1
CDK18/cyclin Y	18	CDK6 /cyclin D3	3	CK1g2	3	DAPK1	26	DYRK1B	0

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
DYRK2	2	EPHA8	11	ERN2 /IRE2	19	FLT4 /VEGFR3	0	GRK6	25
DYRK3	0	EPHB1	3	FAK /PTK2	38	FMS	1	GRK7	48
DYRK4	3	EPHB2	0	FER	1	FRK /PTK5	0	GSK3a	2
EGFR	1	EPHB3	7	FES/FPS	1	FYN	4	GSK3b	4
EPHA1	7	EPHB4	7	FGFR1	1	GCK /MAP4K2	0	Haspin	20
EPHA2	12	ERBB2 /HER2	1	FGFR2	1	GLK /MAP4K3	0	HCK	3
EPHA3	4	ERBB4 /HER4	1	FGFR3	3	GRK1	84	HGK /MAP4K4	1
EPHA4	0	ERK1	1	FGFR4	3	GRK2	66	HIPK1	4
EPHA5	2	ERK2 /MAPK1	-1	FGR	7	GRK3	14	HIPK2	1
EPHA6	8	ERK5 /MAPK7	1	FLT1 /VEGFR1	1	GRK4	6	HIPK3	1
EPHA7	12	ERK7 /MAPK15	0	FLT3	14	GRK5	39	HIPK4	1

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
HPK1 /MAP4K1	-1	JAK2	5	LCK	2	MAPKAPK3	9	MEKK3	1
IGF1R	3	JAK3	9	LCK2 /ICK	24	MAPKAPK5 /PRAK	1	MEKK6	15
IKKa /CHUK	6	JNK1	36	LIMK1	1	MARK1	4	MELK	3
IKKb /IKBKB	15	JNK2	53	LIMK2	4	MARK2/PAR-1Ba	10	MINK /MINK1	1
IKKe /IKBKE	14	JNK3	70	LKB1	35	MARK3	10	MKK4	35
IR	-1	KDR /VEGFR2	0	LOK/STK10	57	MARK4	3	MKK6	16
IRAK1	1	KHS /MAP4K5	-1	LRRK2	10	MEK1	49	MKK7	20
IRAK4	12	KSR1	3	LYN	3	MEK2	31	MLCK /MYLK	4
IRR/INSRR	60	KSR2	1	LYN B	1	MEK3	75	MLCK2 /MYLK2	1
ITK	1	LATS1	21	MAK	77	MEKK1	4	MLK1 /MAP3K9	14
JAK1	13	LATS2	3	MAPKAPK2	12	MEKK2	0	MLK2 /MAP3K10	-1

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
MLK3 /MAP3K11	2	MST3 /STK24	8	NEK5	0	p70S6K /RPS6KB1	2	PDGFRb	3
MLK4	30	MST4	4	NEK6	-1	p70S6Kb /RPS6KB2	2	PDK1 /PDPK1	11
MNK1	-1	MUSK	-3	NEK7	0	PAK1	49	PEAK1	2
MNK2	1	MYLK3	29	NEK9	0	PAK2	31	PHKg1	23
MRCKa /CDC42BPA	31	MYO3A	67	NIM1	3	PAK3	16	PHKg2	13
MRCKb /CDC42BPB	8	MYO3b	-1	NLK	2	PAK4	2	PIM1	2
MSK1 /RPS6KA5	9	NEK1	1	OSR1 /OXSR1	1	PAK5	8	PIM2	4
MSK2 /RPS6KA4	5	NEK11	2	P38a /MAPK14	13	PAK6	316	PIM3	2
MSSK1 /STK23	1	NEK2	1	P38b /MAPK11	3	PASK	9	PKA	2
MST1/STK4	39	NEK3	1	P38d /MAPK13	9	PBK/TOPK	-1	PKAcb	5
MST2/STK3	5	NEK4	1	P38g	3	PDGFRa	0	PKAcg	2

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
PKCa	30	PKCzeta	0	PLK4/SAK	1	ROS /ROS1	15	SLK/STK2	43
PKCb1	83	PKD2 /PRKD2	2	PRKX	48	RSK1	11	SNARK /NUAK2	79
PKCb2	19	PKG1a	63	PYK2	5	RSK2	2	SRMS	1
PKCd	7	PKG1b	47	RAF1	21	RSK3	3	SRPK1	0
PKCepsilon	3	PKG2 /PRKG2	55	RET	4	RSK4	0	SRPK2	-1
PKCeta	151	PKN1 /PRK1	807	RIPK2	3	SGK1	0	SSTK /TSSK6	4
PKCg	41	PKN2 /PRK2	1110	RIPK3	72	SGK2	-5	STK16	4
PKCiota	4	PKN3 /PRK3	86	RIPK5	1	SGK3 /SGKL	23	STK21/CIT	63
PKCmu/PRKD1	1	PLK1	1	ROCK1	56	SIK1	2	STK22D /TSSK1	1
PKCnu/PRKD3	4	PLK2	-2	ROCK2	29	SIK2	7	STK25 /YSK1	6
PKCtheta	42	PLK3	2	RON/MST1R	-2	SIK3	6	STK32B /YANK2	2

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
STK32C /YANK3	6	TAOK3 /JIK	2	TNK1	1	TYK1 /LTK	1	WNK1	1
STK33	1	TBK1	37	TRKA	0	TYK2	18	WNK2	4
STK38 /NDR1	2	TEC	0	TRKB	1	TYRO3 /SKY	1	WNK3	3
STK38L /NDR2	45	TESK1	2	TRKC	2	ULK1	1	YES/YES1	7
STK39 /STLK3	1	TGFBR2	7	TSSK2	1	ULK2	0	YSK4 /MAP3K19	26
SYK	13	TIE2/TEK	0	TSSK3 /STK22C	2	ULK3	10	ZAK /MLTK	1
TAK1	52	TLK1	55	TTBK1	4	VRK1	70	ZAP70	3
TAOK1	1	TLK2	21	TTBK2	4	VRK2	61	ZIPK /DAPK3	9
TAOK2 /TAO1	2	TNIK	-6	TXK	17	WEE1	14		

Table 2. Kinase screening of orobol (1 μM)

26 kinases were selected by criteria, an effect of inhibiting more than 100 % kinase activity, based on Table 1, And then, kinase profiling analysis charged one more. Data were representative of two independent experiments that offered similar results.

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
ACK1	91	CK1g3	108	MLK2 /MAP3K10	94	PBK /TOPK	74	TNIK	68
BTK	95	ERK2 /MAPK1	110	MNK1	69	PLK2	80	ULK2	104
CAMK1a	98	HPK1 /MAP4K1	103	MUSK	61	RON /MST1R	78		
CAMK1d	90	IR	111	MYO3b	86	SGK2	98		
CDK5/p25	78	KDR /VEGFR2	76	NEK5	85	SRPK2	101		
CK1epsilon	74	KHS /MAP4K5	72	NEK6	79	TIE2/TEK	87		

3.4. Orobol suppresses sUV-induced MAPKs phosphorylation in HaCaT cells and HDFs.

I next investigated the molecular mechanism of orobol in HaCaT cells and HDFs to assure that TOPK was a direct target. TOPK is known as upstream of MAPKs and phosphorylates them. And sUV induces MAPKs signaling pathway and it has an important role in regulating MMPs expression [37]. Thus, I perform kinase assay of MAPKs. As a result, the effect of orobol in sUV-induced MAPKs signaling pathway was evaluated. Orobol decreased sUV-induced MAPKs (ERK, JNK and p38) in HaCaT cells (25 kJ/m^2) and HDFs (35 kJ/m^2). In hence, it did not reduce Akt-p70 signaling pathway in both cells. Judging from the result, TOPK could be potential target kinase of orobol. These results suggested that inhibition of MAPKs (ERK, p38 and JNK) by orobol load to the suppression of AP-1 transactivation.

Figure 3

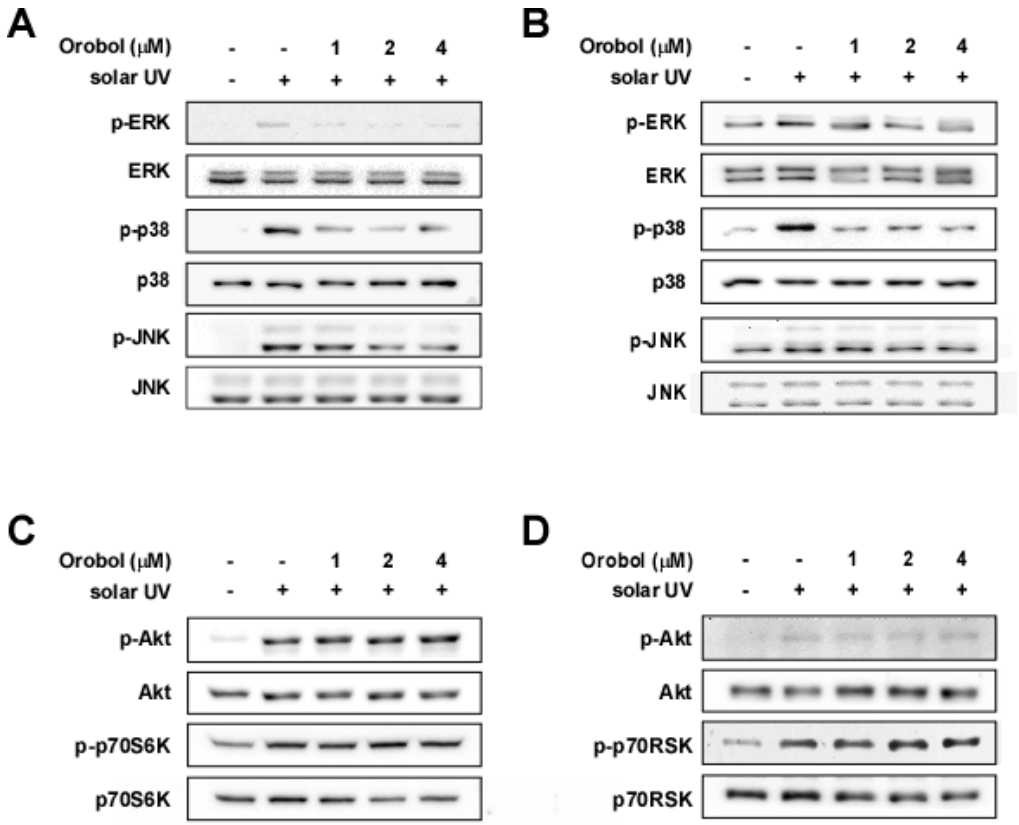
A
Orobol (μM) - - 1 2 4
solar UV - + + + +
p-ERK
ERK
p-p38
p38
p-JNK
JNK
B
Orobol (μM) - - 1 2 4
solar UV - + + + +
p-ERK
ERK
p-p38
p38
p-JNK
JNK
C
Orobol (μM) - - 1 2 4
solar UV - + + + +
p-Akt
Akt
p-p70S6K
p70S6K
D
Orobol (μM) - - 1 2 4
solar UV - + + + +
p-Akt
Akt
p-p70RSK
p70RSK

Figure 3. Inhibitory effect of orobol on sUV-induced signaling pathways.

A and B. Effect of orobol on sUV-induced phosphorylation of MAPKs (ERK, p38, JNK) signal in HaCaT cells (*A*) and HDFs (*B*). **C and D.** Effect of orobol on sUV-induced phosphorylation of Akt-p70 signaling pathway in HaCaT cells (*C*) and HDFs (*D*). After orobol treatment and sUV irradiation (25 kJ/m^2 on HaCaT cells and 35 kJ/m^2 on HDFs), the cells were lysated as described in the Materials and Methods. Phosphorylated and total form of indicated proteins were determined by Western blotting as described in the Materials and Methods. Total forms of each protein were used as a loading control.

3.5. Orobol inhibits TOPK activity and directly binds to ATP non-competitive binding site of TOPK.

Orobol markedly inhibited TOPK kinase activity with the half maximal inhibitory concentration (IC_{50}). 1.67 μM was shown as IC_{50} (Fig. 4A). To determine if the inhibition of TOPK activity by orobol was caused by direct interaction, *in vitro* pull-down assay using active TOPK was performed. As a result, I confirmed that TOPK directly bind to orobol (Fig. 4B). Also, I inspected direct binding interaction between orobol and TOPK in cell lysate (Fig. 4C). Consequently, orobol was directly combined with TOPK, decreasing its kinase activity. Furthermore, I observed whether the binding between TOPK and orobol was ATP competitive or not, through ATP competition binding assay. As a Fig. 4D, orobol suppressed TOPK activity in an ATP non-competitive manner. In summary, orobol directly bound to TOPK as promoting the inhibition of TOPK kinase activity.

Figure 4

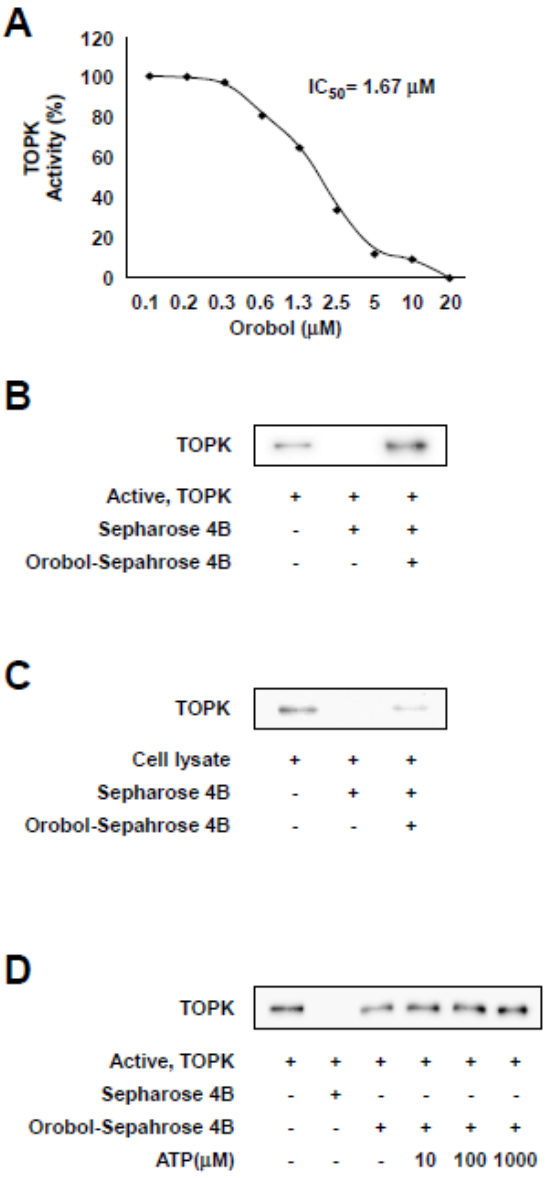
A
120
100
80
60
40
20
0
TOPK Activity (%)
IC50= 1.67 μM
0.1 0.2 0.3 0.6 1.3 2.5 5 10 20
Orobol (μM)
B
TOPK
Active, TOPK + + +
Sepharose 4B - + +
Orobol-Sepahrose 4B - - +
C
TOPK
Cell lysate + + +
Sepharose 4B - + +
Orobol-Sepahrose 4B - - +
D
TOPK
Active, TOPK + + + + + +
Sepharose 4B - + - - - -
Orobol-Sepahrose 4B - - + + + +
ATP(μM) - - - 10 100 1000

Figure 4. Inhibitory effect of orobol on TOPK activity and direct binding between orobol and TOPK.

A. Ten concentrations of orobol were tested for inhibitory activity with 2-fold serial dilutions starting at 20 μM in the presence of TOPK as described in the Materials and Methods. **B.** Orobol directly bound with TOPK. Orobol binding was evaluated by immunoblotting using an antibody against TOPK. Lane 1 (input control), active TOPK; lane 2, active TOPK precipitated with Sepharose 4B, and lane 3, active TOPK precipitated with orobol-Sepharose 4B. **C.** Orobol directly interacted with TOPK in cell lysate. The binding orobol with HaCaT cell lysate was evaluated by immunoblotting; Lane 1, whole cell lysate from HaCaT cells; lane 2, lysate precipitated with Sepharose 4B; lane 3, whole cell lysates form HaCaT cells precipitated by orobol-Sepharose 4B beads. **D.** Active TOPK was incubated with ATP at different concentrations (10, 100 and 1000 μM) for 1 h, and 100 μl of orobol-Sepharose 4B or Sepharose 4B (as negative control) were added. Lane 1, (input control), active TOPK; lane 2, active TOPK precipitated with Sepharose 4B; lane 3, active TOPK precipitated with orobol-Sepharose 4B; lane 4, 5 and 6, active TOPK precipitated with orobol-Sepharose 4B with different concentrations of ATP.

Figure 5

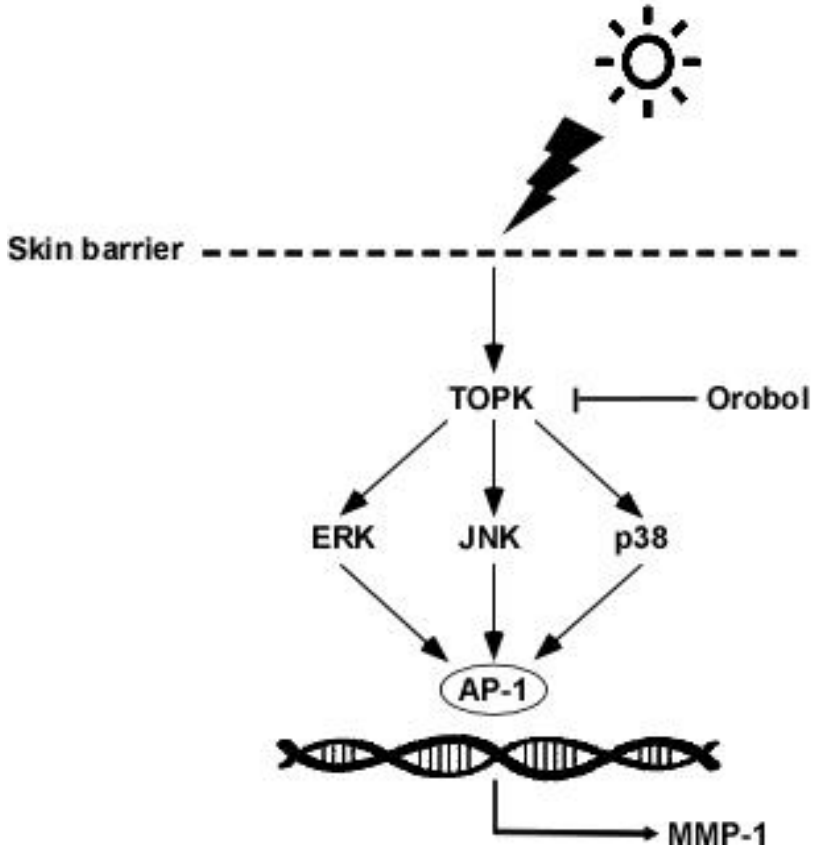

Figure 5. Proposed mechanism of orobol.

Orobol inhibited on sUV-induced MMP-1 expression by directly suppressing TOPK activity in HaCaT cells and HDFs.

Ⅳ. DISCUSSION

Photoaging is the term calling wrinkle, resulting from exposure to outdoor primarily UV irradiation. Chronic sun exposure is a major cause of wrinkles, collagen and elastic fiber changing in the epidermis and dermis. The ultraviolet that penetrate ozone layer and reach the earth's surfaces is consisted of generally 95% UVA (320–400 nm) and generally 5% UVB (280–320 nm) [9]. UVA is considered as the "aging ray" involving in skin photo-aging, participated in the long term clinical effects. UVB containing more energy than UVA rapidly induces acute wrinkle, sun burn and skin cancers [38, 39]. Thus, it is important to use sUV wavelengths with consist UVA and UVB to understand biological effects in deep dermis and both short and long photo-aging [40]. After chronical exposure to the sUV, epidermis become shorter and thicker and degenerative changes are occurred. In hence, loss of cross-linking of collagen is increases in dermis [15]. Additionally, It causes various skin diseases such as erythema, photo-immunosuppression, skin cancers and photo-aging [8, 40]. To understand physiological effect in skin aging, I used sUV wave length like actual sUV, consisting both UVA and UVB. I also investigated both HaCaT cells, predominant cell type in the epidermis, and HDFs abundantly within the dermis. All experiences are performed in two

doses, 25 kJ/m^2 on HaCaT cells and 35 kJ/m^2 on HDFs. The degrees are that the marker gene MMP-1 is well expressed and the cells does not die. It corresponded respectively to 1 or 2 h of the UV daylight dose in New York, USA [40].

In this study, orobol was shown to inhibit sUV-induced MMP-1 expression in HaCaT cells and HDFs through decreasing AP-1 transactivation. And I found out that an anti-photoaging effect of orobol was superior to its precursor, genistein and most effective among isoflavones (genistin, daidzein, daidzin, 6-ODI, 3'-ODI, 8-ODI and equol).

Soybean is an attractive source of cosmetics and foods because of safety and bioactivity. For this reasons, soybean ingredients like soybean fiber, peptide and isoflavones are used as a cosmetic ingredients [41]. To produce an ingredient with better functionality, enzymes for fermentation have been used for bioconversion. Orobol is one of the derivatives converted from genistein which is a most abundant isoflavone in soybean [25]. Orobol exists slightly in tempeh which is fermented soybean [42]. But limitation of the contents, only few researches and industrial usage had been accomplished [31, 33, 34]. Recently, bioconversion strategy of producing orobol has been studied via using tyrosinase as a monophonol monooxygenase. The regio-selective-hydroxylation of genistein was performed by whole *E. coli* cell

biotransformation with heterologously expressed tyrosinase from *Bacillus megaterium* [32].

Orobol is hydroxylated ingredients with genistein. It has been known that it has anti-cancer effect for human ovarian and breast cancer [34, 46]. But improved bioactivity including skin anti-aging effect of orobol has never been studied. As previous studies, hydroxylation in phytochemical increases biological activity in human. For instances, quercetin hydroxylated form of kaemferol in red wine exerted the improved chemoprevention effect in mouse skin cells [43]. In case of soybean isoflavones, 3'-ODI is hydroxylated product of daidzein as a result of fermentation in soybean. After conversion, It has more effects on cancer protectant activities skin whitening and skin wrinkle, which highlighted as a multifunctional bioactive substance in industry [44]. 6-ODI, also bioconversion of daidzein, is revealed a better inhibitory effect on MMP-1 expression than precursor daidzein as an industrial anti-wrinkle ingredient [45]. Because of addition of hydroxyl group at the 3' position of chemicals, its interactions with kinases could be stronger. The hydroxyl group could form a hydrogen bond with the backbone carbonyl group. And the hydrophobic site acts as the inhibitor sandwiched in the binding site [43,44]. Hydroxyl group is also added to genistein to make orobol at the 3'position of genistein. Thus, orobol is expected to interact stronger with the hinge region of TOPK than that of genistein and orobol could be effectively inhibit TOPK

kinase activity. In conclusion, orobol is expected to see an increase in anti-wrinkle effects because of hydroxylation.

In this study, I found that TOPK is a direct molecular target of orobol on sUV-induced MMP-1 expression. Direct inhibition of TOPK by orobol reduced sUV-induced MAPK signaling and MMP-1 expression. TOPK, a member of MAP kinase kinase family, has been implicated in various biological activity, such as cell and tissue proliferation, migration, survival, and death [18, 36, 47]. According to previous studies, TOPK constitutively activates MAPKs. It stimulates mitogen-activate protein kinase signaling, that lead to increased level of phosphorylated ERK and transformation as a potential cancer therapeutic target [21]. And TOPK is necessary for appropriate activation and function of the p38 pathway by growth, acting different growth factors and reducing cell motility [19, 36]. And in UV-induced condition, TOPK participates in JNK activation, interacting with JNK-interacting protein 1. Furthermore, TOPK plays a pivotal role in JNK1-mediated cell transformation induced by UVB [36]. Taken together, these findings show that TOPK positively acts as modulator of sUV-induced MAPKs, major signaling pathways that related to transactivation of AP-1. These result raises the possibility that TOPK might be a potential target of skin photoaging.

In further study, MMP-1 expression and MAP kinase signaling

pathway in TOPK knocked-down HaCaT cells and HDFs should be investigated, to confirm the relation of TOPK and sUV-induced skin photoaging. Furthermore, although datas in this paper support the biological activity and mechanisms of orobol *in vitro*, skin equivalent models and clinical studies are required to further validate its anti-skin aging effect.

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Ⅵ. 국문초록

콩 내의 생리활성 물질인 이소플라본은 주름억제, 미백, 보습 등의 효능을 갖기에 유용한 기능성 화장품, 식품 소재이다. 본 연구에서는 콩 이소플라본 genistein의 대사체인 오로볼이 피부 노화를 예방할 수 있음을 제시하였다. 광노화는 자외선 노출에 의해 비정상적으로 유도된 콜라겐 분해효소 (Matrix metalloproteinase, MMP-1)가 피부 내 콜라겐을 과다하게 분해하는 과정이다. MMP-1의 분비는 자외선에 의해 활성화된 세포 전달 기작이 MMP-1의 상위 전사인자인 Activator protein-1 (AP-1)의 전사과정을 촉진함으로써 일어난다. 본 연구 결과, 콩 발효 대사체인 오로볼은 피부 각질 형성세포 및 인간 진피 섬유아세포에서 자외선에 의해 유도된 MMP-1의 단백질 발현을 농도 의존적으로 억제하였으며, MMP-1 전사 과정 역시 저해됨을 확인하였다. 또한, 오로볼은 MMP-1의 주요 전사인자인 AP-1을 효과적으로 억제하였다. AP-1 및 MMP-1을 조절하는 상위 신호전달 표적 단백질을 규명하기 위하여 인체 내 존재하는 359종의 Kinase와의 결합을 확인하는 Kinase profiling을 수행하였다. 그 결과, T-LAK Cell-originated Protein Kinase (TOPK)를 orobol과 직접 결합하는 상

위 신호전달 표적 단백질로 선정하였다. kinase assay와 pull-down assay를 수행한 결과, 오로볼은 TOPK에 직접적으로 결합함으로써 MAPKs (ERK, JNK, p38)의 인산화로 인한 AP-1 활성을 막는 것을 규명하였다. 추후, TOPK를 knock-down한 세포에서 동일한 신호 기작이 일어나는지에 대한 추가 연구가 진행될 예정이다.

본 연구 결과를 통해 오로볼은 자외선에 유도된 피부노화를 억제하는 강력한 화학적 효능을 가지고 있음을 규명하였다. 본 연구를 바탕으로 오로볼을 자외선 노출에 의해 발생되는 피부 노화를 개선할 수 있는 소재로 이용할 수 있을 것이다.