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Efficacy Validation of Synthesized Retinol Derivatives In Vitro: Stability, Toxicity, and Activity

Hye-Sook Han,[a,b] Youn-Ja Kwon,[b] Myoung-Soon Park,[b] Si-Ho Park,[b] So-Mi Kim Cho,[b] Young-Soy Rho,[c] Jin-Wou Kim,[d] Hong-Sig Sin[b,*] and Soo-Jong Um[a,*]

[a]*Department of Bioscience and Biotechnology/Institute of Bioscience, Sejong University, Seoul 143-747, South Korea*
[b]*Chebigen Inc., 305-B, Chungmugwan, Sejong University, Seoul 143-747, South Korea*
[c]*Department of Chemistry, Chonbuk National University, Chonju 561-756, South Korea*
[d]*Department of Dermatology, St. Paul's Hospital, Catholic University, Seoul 130-709, South Korea*

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Abstract—Retinol (vitamin A) is used as an antiwrinkle agent in the cosmetics industry. However, its photo-instability makes it unsuitable for use in general cosmetic formulations. To improve the photo-stability of retinol, three derivatives (**3**, **4**, and **5**) were synthesized and their biological activities were analyzed. ^{1}H NMR and HPLC analysis indicated that derivatives **3** and **5** were much more stable than retinol under our sunlight exposure conditions. When human adult fibroblasts were treated, the IC_{50} of derivative **3** was 96 μM, which is similar to that of retinol, as determined by the MTT assay. Derivatives **4** and **5** were 2.5 and 8 times more toxic than retinol, respectively. At 1 μM treatment, like retinol, derivatives **3** and **4** were specifically active for RARα out of six retinoid receptors (RAR/RXRα, β, γ). Dose-dependent analysis confirmed that derivative **4** was as active as retinol and the other two derivatives were less active for RARα. The effect of our derivatives on the expression of collagenase, an indicator of wrinkle formation, was measured using the transient co-expression of c-Jun and RT-PCR in HaCaT cells. Collagenase promoter activity, which is increased by c-Jun expression, was reduced 42% by retinol treatment. The other derivatives inhibited collagenase promoter activity similarly. These results were further confirmed by RT-PCR analysis of the collagenase gene. Taken together, our results suggest that retinol derivative **3** is a promising antiwrinkle agent based on its higher photo-stability, lower RARα activity (possibly indicating reduced side effects), and similar effect on collagenase expression.

Introduction

Photo-aging is premature skin aging caused by continuous exposure of the skin to solar UV irradiation. It results in several skin symptoms, such as leathery texture, mottled pigmentation, and wrinkles.[1,2] This irreversible process largely results from UV induction of matrix metalloproteinases (MMPs) that degrade skin collagen. Of MMPs, MMP-1 (collagenase) is mainly involved.[3] UV exposure leads to phosphorylation of c-Jun and ATF-2 transcription factors via activation of the JNK and p38 pathways.[4,5] Once phosphorylated, c-Jun and ATF-2 further induce the expression of c-Jun at the mRNA level. Both activated and induced c-Jun bind to the collagenase gene promoter and increase collagenase expression.[6,7] Therefore, actively increased c-Jun is a critical mediator of the expression of collagenase, which causes aged skin.

Retinol is a hydrophobic vitamin A compound that exerts a potent influence on cell differentiation, proliferation, homeostasis, and development.[8] A physiological metabolite of retinol is all-*trans*-retinoic acid (RA). On entry into cells, retinol is converted into all-*trans*-RA or 9-*cis* RA, which translocate to the nucleus where they bind to RAR or RXR. Both receptors are ligand-inducible transregulators that modulate the transcription of the target genes by associating with *cis*-acting RA response elements (RARE).[9] In addition to activating its receptor, RA interferes with the transactivation function of activation protein-1 (AP-1), which is composed of c-Jun and c-Fos.[10,11] The RA-dependent interference with AP-1 may inhibit the expression of MMPs, such as collagenase (MMP-1) and stromelysin (MMP-3).[12,13] These functions make retinol and its metabolite RA promising cosmetic agents for preventing

*Corresponding authors. Tel.: +82-2-340-83641; fax: +82-2-340-83334. E-mail: umsj@sejong.ac.kr (S.-J. Um); hssin@chebigen.com (H.-S. Sin).

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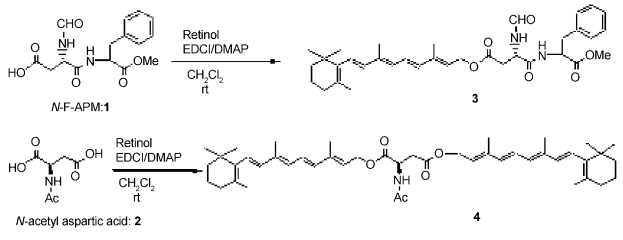
skin aging.[14–16] Since direct topical treatment with RA causes severe side effects, such as skin irritation, cosmetic use of RA is limited.[17,18] Retinol is an alternative for long-term treatment, although its anti-aging activity is lower. However, its photo-instability makes it less useful in general cosmetic formulations.[19] To solve these problems, many groups have synthesized retinol derivatives,[20] blended retinol with soybean oil,[21] or micro-encapsulated retinol.[22] One retinol derivative, retinyl palmitate, has been used in cosmetics for years.[23,24] However, it is considered the least effective topical retinol derivative because it needs to be converted to active RA via a two-step oxidative process that may be limited in skin.[25] Therefore, it remains a challenge to synthesize more retinol derivatives that have higher activity and stability, and which cause less local irritation and toxicity.

In this study, we synthesized retinol derivatives and analyzed whether these compounds are better anti-aging agents than retinol. Our biological assays indicated that retinol derivative **3** is a promising antiwrinkle agent based on its higher photo-stability, lower effect on RARα (possibly reflecting reduced irritation), and similar effect on collagenase expression.

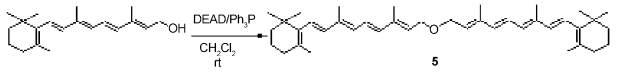
Results and Discussion

Chemistry

The synthesis of retinol derivatives **3–5** is illustrated in Schemes 1 and 2. Briefly, all-*trans* retinol was prepared in 98% yield by reacting commercially available all-*trans* retinyl acetate (Sigma, St. Louis, MO, USA) with potassium carbonate powder.[1] *N*-Formyl aspartame **1** was prepared using the method of Takemoto et al.[26] Allowing α-amino acid compounds such as *N*-formyl aspartame **1** or *N*-acetyl aspartic acid **2** to react with all-*trans* retinol, using EDCI/DMAP as reagent, the corresponding retinyl *N*-formyl aspartamate (retinol derivative **3**) and diretinyl *N*-acetyl aspartate (derivative **4**) were obtained, as shown in Scheme 1. Diretinyl ether (derivative **5**) was obtained by coupling two retinol molecules in methylene chloride with DEAD/Ph_3P treatment (Scheme 2).[27]



Scheme 1.



Scheme 2.

Photo-sensitivity

Natural retinol is very unstable under sunlight and decomposes or isomerizes readily. To prevent this, research groups have tried synthesizing retinol derivatives, blending retinol with peanut oil, or micro-encapsulating retinol. We synthesized three retinol derivatives (**3**, **4**, and **5**), and analyzed their photo-stability by qualitative NMR and quantitative HPLC. For NMR analysis, retinol derivatives were dissolved in 50 mg/mL $CDCl_3$ in NMR tubes. After exposure to sunlight for the number of days indicated, continuous-flow ^1H NMR spectra were obtained. As implied by the olefinic region of the NMR spectra, most retinol was decomposed within 3 h, whereas derivatives **3**, **4**, and **5** remained stable for 5, 2, and 30 days, respectively (Fig. 1A). For time-dependent quantification, HPLC analysis was employed. As shown in Figure 1B, retinol was shortly decomposed, while derivatives **3** and **5** remained mostly intact until 15 days under our experimental conditions. Half-life of derivative **4** was about 5.5 days. These data suggest that our synthetic derivatives, specially **3** and **5** are much more stable than retinol, even when mixed

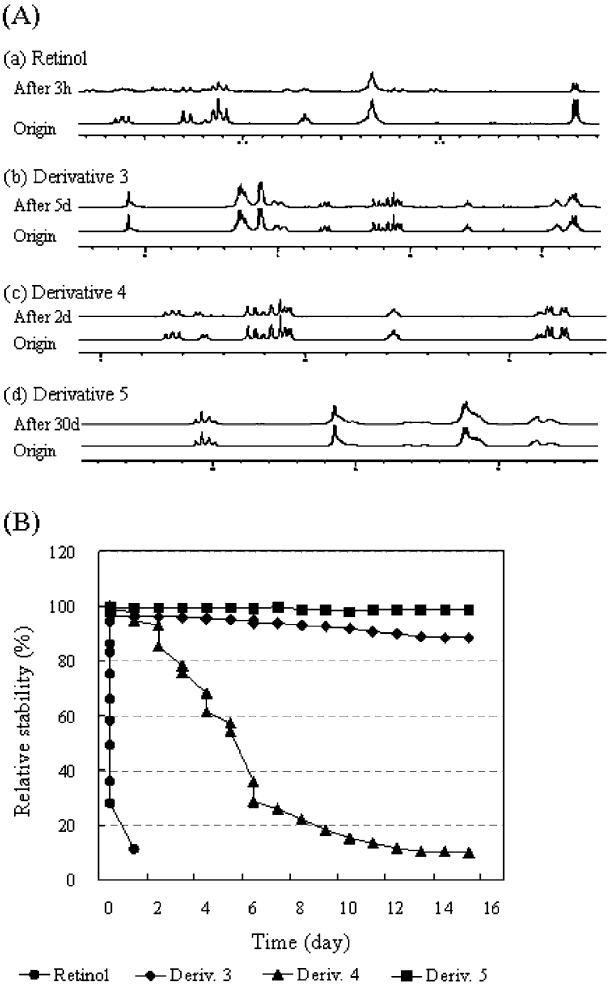


Figure 1. Effect of solar light exposure on the photo-stability of retinol derivatives. Qualitative and quantitative assays were performed by NMR (A) and HPLC (B). Details are described in the Experimental. Stability (%) indicates relative purity of each compound determined by HPLC analysis.

with BHT/BHA antioxidant. Although the data are not shown, our heat stability test indicated that derivative **3** was as stable as retinyl palmitate at 40C for 10 days, as determined by LC–MS analysis.

Cytotoxicity of the retinol derivatives

Human fibroblasts were resistant to up to 100 μM retinol or derivative **3**, whereas the cells were moderately sensitive to derivative **4** and markedly sensitive to derivative **5** at two concentrations treated (Fig. 2A). The cytotoxicity of the derivatives was quantified by the MTT assay. To measure the IC_{50}, the concentration inhibiting growth by 50%, cultured human fibroblasts were treated with each derivative at seven different concentrations (0, 1, 5, 10, 50, 100, 500 μM). As shown in Fig 2B, the IC_{50} of retinol and derivative **3** was 96 μM, whereas the IC_{50} of derivatives **4** and **5** was 38 and 12 μM, respectively. These results indicate that the cytotoxicity of derivative **3** is similar to that of retinol. Since derivative **3** is more stable under sunlight than retinol and is as stable as retinyl palmitate at 40 °C for 10 days, it may be a more effective antiwrinkle agent in cosmetic formulations.

Effect of retinol derivatives on RARα

The transcriptional activity of retinol derivatives on their receptors was determined using skin keratinocyte-derived HaCaT cells that were transiently transfected

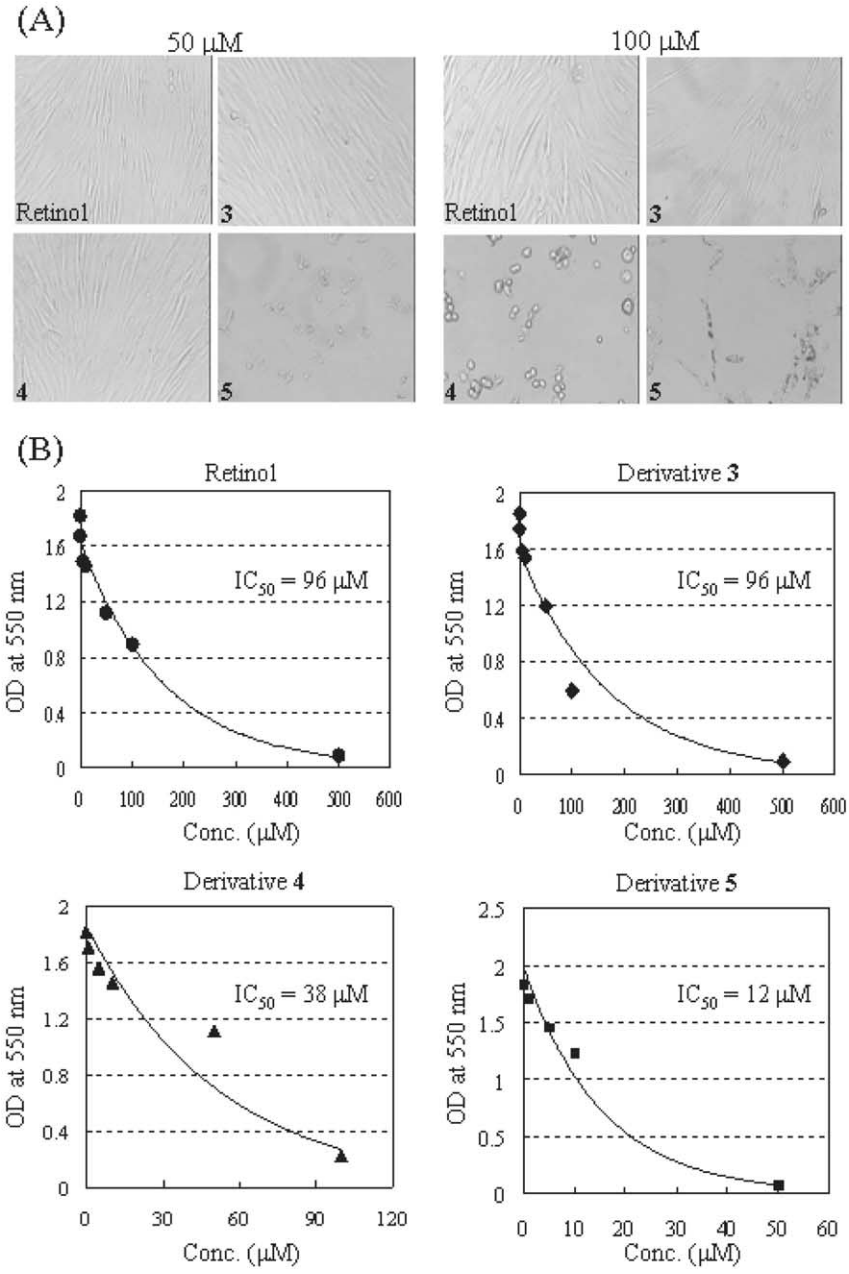


Figure 2. Cytotoxic effect of retinol derivatives. (A) Human adult fibroblast cells (P3) were treated with 50 μM (left) or 100 μM (right) of retinol derivatives for 48 h, and photographed with phase-contrast microscope at ×400. (B) After cells were treated with each derivative at seven different concentrations (0, 1, 5, 10, 50, 100, 500 μM) for 48 h, the IC_{50} values of derivatives were determined by the MTT assay.

with DR5-tk-CAT for RAR (α, β, or γ) and with DR1-tk-CAT for RXR (α, β, or γ). The CAT ELISA was used to measure the amount of CAT protein induced in response to retinol derivatives. When 1 μM of compound was treated, all-*trans*-retinoic acid (RA) was most active for all three RARs, while retinol and derivative **4** was little less active with specificity on RARα (Fig. 3A). In addition, derivative **3** retained some activity selectively for RARAα, but derivative **5** was completely inactive. As expected, all compounds except 9-*cis* RA were inactive for RXRs (Fig. 3B). Our dose-dependent assays confirmed that derivative **4** was as active as retinol for RARα, whereas derivative **3** was less active and derivative **5** was not active (Fig. 3C).These results suggest that like retinol, our derivatives **3** and **4** have selective activity on RARα. Since greater RAR and RXR activity and lack of subtype-specificity are indicative of side effects, such as skin irritation,[17,18] retinol derivative **3** appears to be less likely to cause skin irritation.

Effect of retinol derivatives on c-Jun and collagenase expression

RARs activated by ligand (binding RA, retinol or their derivatives) can functionally interact with the transcription factor c-Jun and inhibit its activity.[10–13] This mechanism of transrepression is critical, since it allows RA or retinol to directly interfere with the photo-aging

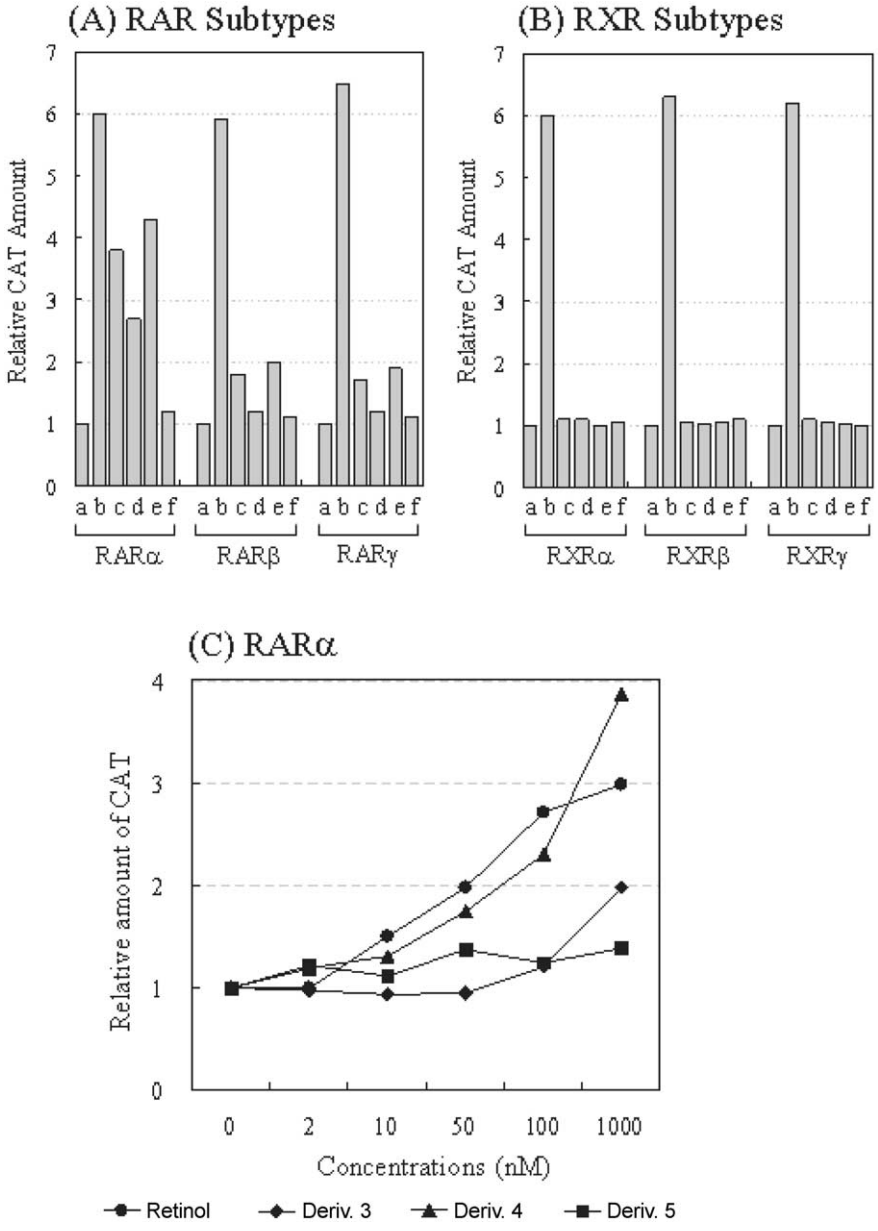


Figure 3. Effect of retinol derivatives on the transcriptional activity of RARα. HaCaT cells were cotransfected with reporter gene DR5-tk-CAT for RARs (A) or DR1-tk-CAT for RXRs (B) and the plasmids expressing each subtype of RARs or RXRs (α, β, γ). After transfections, cells were treated with 1 μM of retinol or each derivative (A, B), and by increasing its concentration (C). The effects of DMSO (a), all-*trans* RA or 9-*cis* RA (b), retinol (c), derivative 3 (d), derivative 4 (e), and derivative 5 (f) were examined. All-*trans* RA and 9-*cis* RA were used as positive controls for RARs and RXRs, respectively. Transcriptional activities were analyzed using CAT ELISA and expressed as a relative activity compared with that of the DMSO control. The data given in the text are the means of at least three independent transfections. p value was <0.05.

signals mediated by c-Jun. Therefore, we compared the transrepression of retinol with that of the synthetic retinol derivatives by transient transfection in HaCaT cells and CAT ELISA. Transient transfection was performed by cotransfection of the c-Jun-responsive collagenase promoter-CAT reporter and c-Jun expression vectors. As shown in Figure 4A, all three derivatives repressed c-Jun activity as much as retinol, when compared with controls. RT-PCR using primers specific for the collagenase gene further confirmed the anti-c-Jun activity of the retinol derivatives. Consistent with the transfection data, all three derivatives effectively suppressed expression of the collagenase gene as much as retinol in HaCaT cells (Fig. 4B). Recently, great progress has been made in screening dissociating retinoids, which can distinguish the activation of retinoid receptors from the transrepression of c-Jun, since most of the side effects of RA and retinol result from the activation of retinoid receptors.[28–33] In this regard, our retinol derivative **3** might be a dissociating retinoid that is less effective for RAR activation, but actively represses c-Jun. Since the toxicities of retinol and derivative **3** to normal fibroblast cells are similar and the derivative is much more stable than retinol, it might replace retinol as an anti-wrinkle agent in cosmetic formulations.

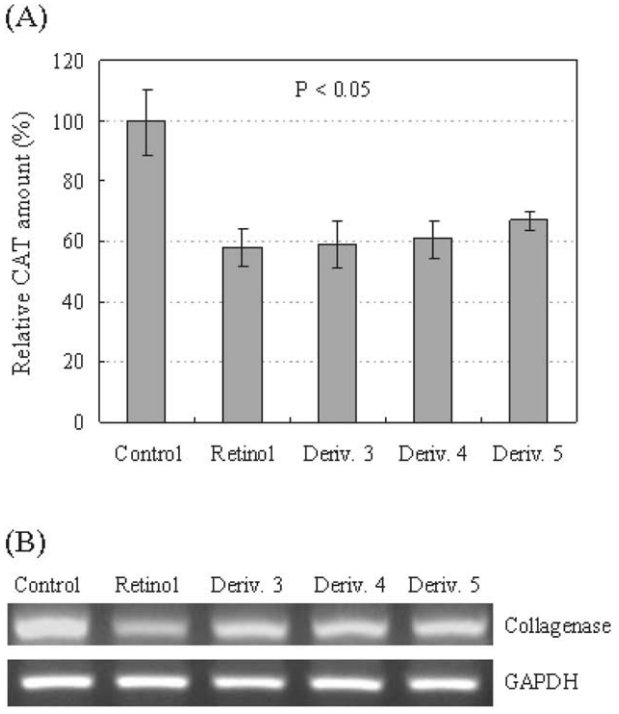


Figure 4. Effects of retinol derivatives on the expression of collagenase gene. (A) Coll-CAT reporter assay. HaCaT cells were cotransfected with Coll-CAT reporter gene and c-Jun expression vector in pSG5, and treated with 1 μM retinol or each derivative. Transcriptional activities were analyzed using CAT ELISA and expressed as a relative activity compared with that of the DMSO control. (B) RT-PCR analysis. Collagenase mRNA expression was determined in HaCaT cells treated with 2 μM retinol derivative for 48 h. GAPDH was used as an internal control.

Conclusion

We synthesized retinol derivatives and analyzed whether these compounds are suitable for use as antiwrinkle agents in cosmetics, using in vitro biological assays. The photo-stability analysis indicated that retinol derivative **3** is much more stable than retinol when exposed to solar radiation. The toxicity of derivative **3** on human fibroblasts was similar to that of retinol, as determined by the MTT assay. Third, transcription assays followed by transient transfection of RARs or RXRs showed that derivatives **3** and **5** are less active with specificity on RARα than retinol or derivative **4**, suggesting that derivatives **3** and **5** could be less irritating. Fourth, retinol and all three derivatives have similar effects on collagenase expression as determined by two independent assays: c-Jun transfection and RT-PCR analysis. Overall, these results suggest that retinol derivative **3** could be a promising antiwrinkle agent for use in cosmetic formulations.

Experimental

Chemistry

All the solvents were carefully dried and distilled following published procedures. All reactions were conducted under an Ar atmosphere, except for those using water as a solvent, which were monitored by TLC on Silica Gel 60 F254 (Merck, Darmstadt, Germany). All products were purified by flash column chromatography on Silica Gel 60 (Merck, 230–400 mesh). UV–vis absorption spectra were recorded on a Shimadzu SPD-10AVP spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL *JNM* EX-400 using $CDCl_3$ as the solvent. All chemical shifts (δ) are quoted in ppm downfield from TMS and the coupling constants (*J*) are given in Hz. Mass spectra were measured on a Shimazu GCMS-PO 1000 mass spectrometer (EI 70 eV).

Stability test

For qualitative analysis of photo-stability, ^{1}H NMR was employed. Samples were prepared in 25 mg/0.5 mL $CDCl_3$ in NMR tubes sealed with Ar gas. After exposed to solar light for the indicated times, the continuous-flow ^{1}H NMR spectra of retinol derivatives were recorded on a JEOL *JNM* EX-400 using $CDCl_3$ as the solvent. For quantitative analysis of photo-stability, HPLC was performed using a Shimadzu Model LC-6AD ternary solvent delivery system. Samples were prepared as 100-μM solutions with $CDCl_3$ in transparent glass vials sealed with Ar gas. Aliquots (50 μM) were loaded into a 5 μm Zorbax SB-C18 column (4.6×150 mm) with a 20 mm guard column. Following elution with methanol/water (93:7, v/v; derivatives **3**, **5**) or methanol only (derivatives **4**) for 15 min at a linear gradient of 1 mL/min, absorption spectra were recorded at 325 nm (derivatives **3**, **4**) and 280 nm (derivative **5**). β-ionone was used as a standard.

(2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetra-enyl(3*S*)-4-[(1-benzyl-2-methoxy-2-oxoethyl)amino]-3-formylamino-4-oxobutanoate (3). To a stirred solution of *N*-F-APM (843.3 mg, 2.6 mmol) in CH_2Cl_2 (5 mL) and DMF (1 mL) was added dropwise a solution of EDCI (683.0 mg, 3.6 mmol) in CH_2Cl_2 (1 mL) at 0 °C. After the mixture had been stirred for 30 min, a solution of retinol (500.0 mg, 1.8 mmol) in CH_2Cl_2 (2 mL) was added via cannulation with the appropriate amount of DMAP. The reaction mixture was stirred for 3 h and CH_2Cl_2 was added (20 mL). The organic layer was washed with water (20 mL×3), dried with $MgSO_4$, and the solvent was evaporated. The crude residue was purified by flash column chromatography on silica gel (eluent = hexane/EtOAc = 2/1) to give **3** (0.87 g, 82%) as a light yellow powder. UV (λ_{max}, DMSO) 329 nm; ^{1}H NMR ($CDCl_3$) δ 8.13 (s, 1H, CHO), 7.28 (m, 3H, ArH), 7.13 (d, 2H, J = 6.84, ArH), 7.01 (d, 1H, J = 17.09, CH=), 6.99 (d, 1H, J = 17.09, CH=), 6.65 (dd, 1H, J = 15.14, 11.23, CH=), 6.26 (d, 1H, J = 15.14, CH=), 6.13 (dd, 1H, J = 16.11, 11.23, CH=), 6.08 (d, 2H, J = 6.35, CH_2O), 5.57 (t, 1H, J = 6.35, CH=), 4.89 (m, 1H), 4.76 (m, 3H), 3.70 (s, 3H, OCH_3), 3.15 (dd, 1H, J = 14.16, 13.67), 3.05 (dd, 1H, J = 14.16, 13.67), 2.97 (dd, 1H, J = 17.09, 3.91), 2.65 (dd, 1H, J = 17.09, 6.84), 2.02 (m, 2H, CH_2), 1.96 (s, 3H, CH_3), 1.88 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 1.61 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.03 (s, 6H, CH_3×2); ^{13}C NMR ($CDCl_3$) δ 171.79, 171.34, 169.55, 160.88, 139.51, 137.75, 137.5, 136.77, 135.56, 135.52, 129.81, 129.38, 129.23, 128.56, 127.17, 127.10, 126.05, 123.59, 62.10, 53.59, 52.37, 47.58, 39.56, 37.60, 35.68, 34.21, 33.02, 28.92, 21.69, 19.21, 14.15, 12.74, 12.71; MS (EI, 70 eV) m/z (relative intensity %) 91(60), 167(100), 268(15), 590(35, M^+); HRMS (EI, M^+) calcd for $C_{35}H_{46}N_2O_6$: 590.3356. Found: 590.3360.

Di[(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatet-raenyl]-2 (acetylamino) succinate (4). Yield 0.62 g (50%) as a light yellow oil. UV (λ_{max}, DMSO) 385 nm; ^{1}H NMR ($CDCl_3$) δ 6.65 (dd, 2H, J = 15.14, 11.23, 2×CH=), 6.26 (d, 2H, J = 15.14, 2×CH=), 6.18 (d, 2H, J = 16.11, 2×CH=), 6.10 (d, 2H, J = 16.11, 2×CH=), 6.09 (d, 2H, J = 11.23, 2×CH=), 5.57 (t, 2H, J = 7.32, 2×CH=), 4.86 (m, 1H, CH–NHAc), 4.81 (d, 2H, J = 7.23, OCH_2), 4.74 (d, 2H, J = 7.32, OCH_2), 3.03 (dd, 1H, J = 17.10, 4.39, CH_2), 2.86 (dd, 1H, J = 17.10, 4.39, CH_2), 2.03 (s, 6H, 2×CH_3), 2.02 (m, 4H, 2×CH_2), 1.95 (s, 6H, 2×CH_3), 1.87 (s, 6H, 2×CH_3), 1.71 (s, 6H, 2×CH_3), 1.61 (m, 4H, 2×CH_2), 1.46 (m, 4H, 2×CH_2), 1.02 (s, 12H, 4×CH_3); ^{13}C NMR ($CDCl_3$) δ 170.96, 170.65, 169.78, 139.80, 139.58, 137.75, 137.52, 136.83, 135.49, 135.47, 129.82, 129.79, 129.39, 127.13, 127.10, 126.11, 126.08, 123.64, 123.43, 62.59, 61.81, 48.61, 39.56, 36.40, 34.22, 33.04, 28.93, 23.16, 21.71, 19.22, 12.75, 12.73; MS (EI, 70 eV) m/z (relative intensity %) : 91 (32), 167 (100), 669 (28), 712 (M^+, 52); HRMS (EI, M^+) calcd for $C_{46}H_{65}NO_5$: 711.4863. Found: 711.4869.

(1*E*,3*E*,5*E*,7*E*)-9[(2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenyl]oxy-3,7-dimethyl-1-(2,6,6-trimethyl-1-cyclohexenyl)-1,3,5,7-nonatetraene (5). To a solution of retinol (0.60 g, 2.10 mmol) and DEAD (0.20 g, 1.15 mmol) in CH_2Cl_2 (20 mL) was added Ph_3P (0.30 g, 1.15 mmol) in CH_2Cl_2 (20 mL). After 2 h at room temperature, the mixture was applied to an alumina column and eluted with EtOAc to remove triphenylphosphine oxide and diethyl hydrazinedicarboxylate. The solvent was evaporated in vacuo, and the residue was extracted with EtOAc (20 mL×2). The extract was washed with H_2O, dried with $MgSO_4$, and the solvent was evaporated. The crude residue was purified by flash column chromatography on silica gel (eluent = hexane/EtOAc = 1/1) to give **5** (0.43 g, 74%) as a light yellow oil. UV (λ_{max}, DMSO) 277 nm; ^{1}H NMR ($CDCl_3$) δ 6.05 (q, 4H, J = 16.11, CH=), 5.14 (br s, 6H, CH=), 4.16 (q, 4H, J = 7.32, OCH_3), 1.99 (s, 6H, 2×CH_3), 1.74 (s, 6H, 2×CH_3), 1.68 (s, 6H, 2×CH_3), 1.63 (t, 4H, J = 5.86, CH_2), 1.45 (m, 4H, CH_2), 1.01 (s, 6H, 2×CH_3), 1.00 (s, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$) δ 137.45, 136.80, 128.87, 62.92, 61.12, 39.49, 34.14, 32.86, 28.84, 21.62, 20.35, 19.20, 14.41, 12.66; MS (EI, 70 eV) m/z (relative intensity %) : 91 (24), 167 (100), 271 (9), 554 (M^+, 41); HRMS (EI, M^+) calcd for $C_{40}H_{58}O$: 554.4488. Found: 554.4491.

Cell culture and MTT growth assay

Human skin fibroblast cells (passage 3) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), previously inactivated at 56 °C for 20 min. The cytotoxic effects of the retinol derivatives were monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in a dose-dependent manner. Cells were seeded at an initial density of 3000 cells per well in 96-well plates. Medium containing fresh retinol or its derivatives was treated with various concentrations (0, 1, 5, 10, 50, 100, 500 μM) for 48 h. DMSO (0.01%), used as a control, did not affect cell proliferation. Fifty microliters of MTT solution (2 mg/mL in PBS) were added to the cell culture medium. The reaction mixture was incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. MTT solution was aspirated and 150 μL of DMSO were added. The optical density was measured spectrophotometrically at 550 nm. IC_{50} values, the concentration required for 50% growth inhibition, were determined for each retinol derivative.

Transcription assay for RARα and c-Jun

Cells were transfected out using a liposome-based method with Lipofectamine (Gibco-BRL, Gaithersburg, MD, USA), as previously reported.[34] Briefly, HaCaT cells (1×10^6 cells), maintained in DMEM supplemented with charcoal-treated 10% FBS, were plated in 60-mm dishes 5 h before transfection. After overnight transfection with the indicated reporter plasmids (DR5-tk-CAT for RAR, DR1-tk-CAT for RXR, and Coll-CAT for c-Jun: generously donated by Dr. Pierre Chambon, Strasbourg, France), the cells were washed, fed with complete medium if needed, supplemented with retinol or its derivatives as indicated in the text, and further incubated for 24 h. Cell extracts were then prepared and

β-galactosidase activity was determined to normalize transfection. The CAT concentration was tested in 30–70 μL of the clear lysate using CAT ELISA according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

HaCaT cells were grown in 10-cm dishes in the absence or presence of retinol or its derivatives. Total cellular RNA was isolated using an RNeasy Total RNA Purification kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. To obtain the linear range, RNA was diluted from 40 ng per tube to 1/5, 1/10, 1/100, and 1/1000. GAPDH was used as a control. RT-PCR was performed as described previously using the Titan One Tube RT-PCR system (Roche Molecular Biochemicals).[34] Primers used for RT-PCR were 5′-CTGAAGGTGATGAAGCAGCC-3′ (sense) and 5′-AGTCCAAGAGAATGGCCGAG-3′ (antisense) to give 428 bp PCR fragment.

Acknowledgements

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