Synthesis and Protective Effects of Bis[4-][N,N-di-(carboxymethyl)amino]-phenoxylalkane Derivatives on UVA-Induced Production of MMP-1 in Human Skin Fibroblasts

Ling-Yih Hsu,* a Chih-Ying Nien, b Wei-Ming Huang, c Shou-Che Hsu, c and Tsu-Chung Chang* c,d,e

a Department of Biological Science and Technology, China University of Science and Technology; Nangang, Taipei 11581, Taiwan, ROC; b School of Pharmacy, National Defense Medical Center; c Department of Biochemistry, National Defense Medical Center; Neihu, Taipei 11014, Taiwan, ROC; d Department of Biotechnology, Asia University; Taichung 41581, Taiwan, ROC; and e Graduate Institute of Basic Medical Science, China Medical University; Taichung 40447, Taiwan, ROC.

Received January 22, 2014; accepted June 10, 2014

UV-induced matrix metalloproteinase (MMP) production is considered a cause of skin aging. In this study, a number of novel bis[4-][N,N-di-(carboxymethyl)amino]phenoxylalkane derivatives were synthesized and evaluated as UVA-protective agents. These compounds significantly protected human dermal fibroblast (HDF) cells from UVA-induced cytotoxicity and inhibited MMP-1 activation and expression with potency comparable to desferoxamine (DFO). Promoter activity assay indicated that they inhibited MMP-1 expression at the transcriptional level. Further studies revealed that the mechanism of these compounds may include blockage of the UVA-induced activation of the p38/mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways. Together, these results suggest that further development of these compounds may be of interest.

Key words metal chelating agent; UVA-protective agent; ultraviolet irradiation; dermal fibroblast

The skin is the outermost and largest organ of the body. Thus, it is most prone to photodamage as it is directly exposed to sunlight. UV sunlight is composed of invisible wavelengths divided into three types: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). Exposure to UV light is linked to long-term skin damage: UVA and UVB light are largely responsible for skin aging and cancer.1,2 Skin aging is commonly related to increased wrinkling, sagging, and laxity.2 There are two distinct types of aging, intrinsic and extrinsic aging. Intrinsic skin aging is primarily determined by heredity, is associated with augmented fragility and loss of skin elasticity that is not apparent until old age.3 In contrast, the vast majority of the skin cosmetic problems are thought to be caused by extrinsic environmental factors such as repeated exposure to sunlight. Sun-exposed or photoaged skin is coarsely wrinkled and may exhibit dyspigmentation and telangiectasia. Matrix metalloproteinases (MMPs), a class of enzymes responsible for the degradation of extracellular matrix proteins, play important roles in skin inflammation and wound healing.5,6 Increased MMP activity and reduced collagen I expression have been considered a cause of skin aging and are observed in naturally aged and photoaged skin.4,6 Therefore, inhibition of MMP production may prove effective for the treatment of photoaged skin.7,8

Reactive oxygen species (ROS) is considered to be the main contributor to the deleterious effects of UVA-induced skin damage. UVA radiation of skin cells induces immediate release of iron from iron-binding proteins such as ferritin, which exacerbates the skin damaging effects.8,9 Iron may participate in the Fenton reaction to generate the highly reactive hydroxyl radical (·OH), which may cause extensive damages to macromolecules including DNA, lipids, and proteins.9 UVA is also known to significantly stimulate the expression and activation of MMPs.7,9 In addition to the harmful effect of excess iron, catalytic zinc metal in the MMPs is essential for the degradation and remodeling of extracellular matrix proteins which may have profound effect on photoaging. Thus, development of metal chelating agents would be a promising approach in protection of skin cells against UVA-induced damages.11,12

Ethylendiaminetetaacetic acid (EDTA), a polyamino carboxylic acid, is one of the best-known and most widely used metal chelating agents in both analytical chemistry13 and medicine.14,15 Its usefulness results from its chemical structure, which consists of at least two carboxylate sites capable of donating electrons to the metal to which it chelates. For true chelation to occur, the carboxylate site(s) must also be in a position within the chelating molecule such that formation of a ring with the metal ion occurs. The aims of the present study were to examine the protective effects of these novel chelating agents, bis[4-][N,N-di-(carboxymethyl)amino]phenoxylalkane derivatives with four carboxylate sites and a flexible –O(CH2)n–O– linker, on the UVA-induced cytotoxicity and MMP-1 production. Here, we demonstrated that these agents efficiently inhibit UVA-induced MMP-1 expression and cell damage in human dermal fibroblast (HDF) cells. Our study provides a rationale for the use of chelating compounds for the prevention of UVA-induced photoaging and photodamage.

Results and Discussion

Chemistry The synthesis of the target compounds was accomplished through the synthetic pathway shown in Chart 1. The commercially available α,ω-dibromoalkanes, including 1,3-dibromopropane, 1,4-dibromobutane, 1,5-dibromopentane, 1,6-dibromohexane, and 1,8-dibromooctane, were used to build the flexible linkers –O(CH2)n–O– (n = 3, 4, 5, 6, 8), in the target compounds. Thus, the reaction of α,ω-dibromoalkanes (with alkyl chains of varied length) with 4-nitrophenolate

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: hlychit@cc.cust.edu.tw; tcchang@ndmctsgh.edu.tw © 2014 The Pharmaceutical Society of Japan
in the presence of dry \( N,N \)-dimethylformamide at 80°C for 12–24 h provided \( \alpha,\omega \)-bis(4-nitrophenoxy)alkanes (1a–e). Subsequently, using the Zinin reaction, the aromatic nitro group of 1a–e was reduced to aromatic amine 2a–e by treatment with sodium sulfide nonahydrate/sodium hydroxide at reflux temperature for 17 h. N-Alkylation of the aromatic amine in 2a–e with ethyl bromoacetate in the presence of potassium carbonate in ethanol at reflux temperature for 48 h with stirring provided 3a–e. Hydrolysis of the ester group in 3a–e using saturated NaOH solution in tetrahydrofuran at reflux temperature for 15 h yielded 4a–e, the target products containing four carboxylate groups.

**Inhibition of UVA-Induced Cytotoxicity**

Compounds 4a–e were first evaluated for cytotoxicity in HDF cells. As shown in Fig. 1, at a concentration of 10 \( \mu M \), there was no significant effect on cell morphology or viability at 48 h. These results indicated that there was no detectable cytotoxicity from the novel agents on HDF cells in this study.

The protective effect of bis\([N,N\)-di-(carboxymethyl)amino\]phenoxy\)alkane derivatives on HDF cells against UVA-induced damage was examined. In this study, the survival rate of HDF cells was found to decrease dramatically with increased UV doses at 24 h after UVA or UVB irradiation (data not shown). Upon UVA irradiation, 50% of the cells were viable at 48 h after the cells were irradiated with 15 J/cm\(^2\) UVA alone. Exposure of HDF cells to compounds 4a–e significantly protected the cells from UVA-induced cell death. As shown in Fig. 2A and Table 1, the protective rates were 41, 43, 53, 58, and 83% for compound 4a–e, respectively. In contrast to UVA, these compounds exhibited much less or no protective effect on UVB-induced damage. As shown in Fig. 2B, the cell viability was 70% at 48 h after irradiation with 40 mJ/cm\(^2\) of UVB alone. Only compound 4a showed significant protection against UVB-elicited insult (92% viability, \( p < 0.05 \)), and compounds 4b–e exhibited no significant UVB protection in HDF cells. Additionally, the protective efficacy of compounds 4a–e against UVB insult appeared to decrease as the length of the carbon chain between the two \( N,N \)-di-(carboxymethyl)amino\]-phenoxy moieties increased. These results demonstrated that compounds 4a–e exhibit specific protective effects in human HDF cells against UVA-elicited insults.

The different radiation energy in UVA and UVB light...
might account for the differential protective effect of these compounds against UVA and UVB irradiation. UVA radiation, which is lower in energy, induced the immediate release of iron in skin cells. Based on their metal chelating potential, compounds 4a–e could prevent the harmful effect induced by UVA-released ions. UVB radiation has shorter wavelengths and higher energy and thus generates more radicals than UVA radiation. Because this series of compounds lacks radical-scavenging conjugated double bonds, they are less effective in preventing the harmful effect resulting from UVB irradiation.

It is interesting to note that the UVA-protective efficacy of these compounds appears to increase with the carbon chain length in between the two \( \text{N, N-di(carboxymethyl)aniline moieties} \) (Fig. 2A). There are several potential mechanisms to account for the observed pattern of protection efficacy. First, with longer carbon chain, the compounds might display greater structural flexibility to bind metal ions effectively. Second, increased carbon chain length renders the compounds greater hydrophobicity and the deeper the compounds to incorporate into lipid bilayer membrane. Thus, the compounds with longer carbon linker protect cell membrane more effectively from UVA-induced membrane damages. Furthermore, the compounds with increased lipophilic characteristics might also penetrate cell membranes more readily than the shorter chain compounds and display more extensive protective activities.

**Inhibition of UVA-Induced MMP-1 Activation and Gene Expression** The photoaging effect of UV radiation is associated with the induction of MMPs in skin cells. Inhibition of MMP-1 activation and expression has been shown to be important to alleviate UV-induced photo-damage to skin cells. Compounds 4a–e showed significant protection of...
HDF cells from UVA-induced cellular damage. Therefore, the effect of these compounds on MMP-1 activity was examined by zymography, and the MMP-1 protein levels were assessed using Western blot analysis. As shown in Fig. 3, UVA elicited a 2.43- and 5.71-fold rise in the MMP-1 activity and protein levels, respectively. The presence of compounds 4a–e significantly inhibited MMP-1 activation and protein levels on UVA irradiation. With the exception of compound 4e, the compounds 4a, b, d, and e reduced MMP-1 activity by 41, 37, 42, and 39%, respectively, compared to cells exposed to UVA-irradiation alone. Similarly, compounds 4a–e effectively decreased the MMP-1 protein level by 64, 60, 44, 60, and 65%, respectively. These results indicated that these compounds are effective MMP inhibitors by blocking MMP-1 activation and expression in cells irradiated with UVA.

It has been previously shown that UVA induces MMP-1 expression primarily through activation of the MMP-1 gene promoter at the transcriptional level in human cells. To confirm the inhibition of compounds 4a–e on MMP-1 expression, the effect of these compounds on MMP-1 promoter activity was examined. The results shown in Fig. 4 indicated that 4a–e significantly inhibited UVA-induced MMP-1 promoter activity. Compared with cells irradiated with UVA alone, compounds 4a–e inhibited the MMP-1 promoter activity by 59, 76, 60, 84, and 65%, respectively. These results demonstrated these compounds inhibited MMP-1 activation and expression primarily at the transcriptional level. Taken together, our results indicated that these compounds are effective in suppressing MMP-1 activation and expression in human HDF cells, which may be associated with the UVA protective effect of these compounds.

Inhibition of UVA-Activated p38 and c-Jun N-Terminal Kinase (JNK) Pathway Previous studies have shown that UVA activates the p38, JNK, and extracellular signal-regulated kinase (ERK) signal kinase pathways. To analyze the signaling pathways involved in the action of compounds 4a–e, the phosphorylation of signal pathway kinases were examined. In this study, we found that UVA activated the p38 and JNK signaling pathways in human HDF cells by increasing their phosphorylation levels, and no effect on the ERK pathway was observed. As shown in Fig. 5, compound 4e showed no significant effect on the p38 or JNK phosphorylation levels. However, 10 µM compound 4e effectively blocked the UVA-induced phosphorylation of p38 and JNK kinases.

Results shown here suggest that UVA-induced raise in intracellular iron lead to the activation of the p38 and JNK pathways in human HDF cells. Previous studies have shown that the selective activation of p38 and JNK signaling kinases is resulted from the activation of cell surface growth factor receptors and cytokine receptors by excess ROS following UVA irradiation. Iron plays an important role in many cellular processes as it was also shown that iron depletion by iron chelators resulted in apoptotic cell death through activation of p38 and JNK pathways. Thus, it appears that activation of p38 and JNK signaling is closely linked to the intracellular iron level, in iron overload or deficiency. In this study, we found that compound 4e, with concentration of 10 µM, is able to block the UVA-induced p38 and JNK signal activation. However, it is interesting to note that, following UVA irradiation, the phosphorylation level of p38 is further increased in the presence of lower concentration, 5 µM, of compound 4e (Fig. 5, lane 5). These results suggest that these compounds might activate different cellular stress signaling through modulating the intracellular iron or other metal levels. Thus, the cross-talk in between these different signaling pathways in skin cells deserves further investigation.

Taken together, our results suggest that these metal chelating compounds impart a UVA-protective effect by blocking the UVA-induced activation of the p38 and JNK pathways. The p38 and JNK pathways regulate a variety of cellular functions, including the activation and expression of MMP-1 and the inhibition of collagen production. Activation of these signaling pathways may perturb the structural integrity of the skin.
extracellular matrix and damage skin tissues, which in turn causes photoaging. Thus, our results suggest that these compounds effectively prevented cell damage and MMP-1 expression through the inhibition of the UVA-activated p38 and JNK pathways in human HDF cells.

Conclusion
In this study, compounds 4a–e were synthesized, and their protective effects on UV irradiated cells were examined. At a concentration with no apparent cytotoxicity, these compounds prevented HDF cells from UVA-elicited MMP-1 expression and cell death. In addition, we also showed that these compounds inhibited MMP-1 expression by blocking the UVA-induced activation of the p38 and JNK signaling kinases. This study demonstrated that the potential metal-chelating derivatives inhibit UVA-induced MMP-1 expression and prevent inflammatory, photoaging, and damaging effects in HDF cells. The (CH₂)₄-length (n = 3, 4, 5, 6, 8) leading to different structural conformations assumed to be important for their activities and it is suitable for further investigation.

Experimental

General
All reagents were purchased from Lancaster and Merck Co. (Darmstadt, Germany). Melting points (mp) were measured on a BUCHI 530 apparatus and are uncorrected. Merck Art No. 105554 plates precoated with Silica gel 60 containing a fluorescent indicator were used for thin-layer chromatography, and Silica gel 60 (Merck Art No. 109385, 230–400 mesh) was employed for column chromatography. Evaporations were carried out at <50°C using a rotary evaporator at reduced pressure (water aspirator). ¹H- and ³¹C-NMR spectra were obtained with a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Where necessary, deuterium exchange experiments were used to obtain proton shift assignments. Mass spectra were recorded on a JEOL J.M.S-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78°C in the presence of P₂O₅ for at least 12 h unless otherwise specified. Elemental analyses were obtained using a Perkin-Elmer 2400 Elemental Analyzer.

General Procedure for the Synthesis of α,α'-Bis(4-nitrophenoxy)alkane (1a–e) A solution of sodium sulfide nonahydrate (210 mmol) and sodium hydroxide (480 mmol) in water (735 mL) was added to a stirred suspension of compounds 1a–e (30 mmol) in ethanol (270 mL). The mixture was heated at reflux for 17 h and left to stand overnight. The resulting precipitate was collected by filtration and repeatedly washed with H₂O. Recrystallization from ethanol afforded the title compounds.

1,3-Bis(4-nitrophenoxy)propane (2a) Yield: 65%, mp 104–105°C, Rf = 0.10 (AcOEt/n-hexane=1/2). ¹H-NMR (DMSO-d₆, 300 MHz) δ: 6.64 (d, 4H, J=8.4 Hz, ArH), 6.48 (d, 4H, J=8.7 Hz, ArH), 4.58 (s, 4H, NH₂), 3.93 (t, 4H, J=6 Hz, OCH₂), 2.0 (m, 2H, CH₂). EI-MS: m/z 258 (M+). Anal. Calcd for C₁₆H₁₈N₂O₂: C 71.97, H 8.05, N 9.33. Found: C 71.52, H 7.50, N 9.01.

1,4-Bis(4-nitrophenoxy)butane (2b) Yield: 71%, mp 135–136°C, Rf = 0.10 (AcOEt/n-hexane=1/2). ¹H-NMR (DMSO-d₆, 300 MHz) δ: 6.64 (d, 4H, J=9.0 Hz, ArH), 6.49 (d, 4H, J=9.7 Hz, ArH), 4.58 (s, 4H, NH₂), 3.84 (m, 4H, OCH₂), 1.75 (m, 4H, CH₂). EI-MS: m/z 272 (M+). Anal. Calcd for C₁₈H₂₀N₂O₂: C 70.56, H 7.40, N 10.29. Found: C 69.62, H 7.49, N 9.06.

1,5-Bis(4-nitrophenoxy)pentane (2c) Yield: 65%, mp 48–49°C, Rf = 0.20 (AcOEt/n-hexane=1/2). ¹H-NMR (DMSO-d₆, 300 MHz) δ: 6.62 (d, 4H, J=8.7 Hz, ArH), 6.48 (d, 4H, J=9.0 Hz, ArH), 4.56 (s, 4H, NH₂), 3.8 (t, 4H, J=6.3 Hz, OCH₂), 1.67 (m, 4H, CH₂), 1.48 (m, 2H, CH₂). EI-MS: m/z 286 (M+). Anal. Calcd for C₁₉H₂₂N₂O₂: C 71.30, H 7.74, N 9.78. Found: C 71.52, H 7.50, N 10.02.

1,6-Bis(4-nitrophenoxy)hexane (2d) Yield: 71%, mp 141–143°C, Rf = 0.20 (AcOEt/n-hexane=1/2). ¹H-NMR (DMSO-d₆, 300 MHz) δ: 6.62 (d, 4H, J=9.0 Hz, ArH), 6.48 (d, 4H, J=9.0 Hz, ArH), 4.55 (s, 4H, NH₂), 3.79 (t, 4H, J=10.2 Hz, OCH₂), 1.64 (m, 4H, CH₂), 1.41 (m, 4H, CH₂). EI-MS: m/z 300 (M+). Anal. Calcd for C₂₀H₂₄N₂O₂: C 71.97, H 8.05, N 9.33. Found: C 71.87, H 8.12, N 9.01.

1,8-Bis(4-aminophenoxy)-octane (2e) Yield: 56%, mp 129–130°C, Rf = 0.24 (AcOEt/n-hexane=1/2). ¹H-NMR (DMSO-
General Procedure for the Synthesis of the Sodium Salt of α,ω-Bis-[4-[N,N-di-(ethoxy carbonylmethyl)amino]phenoxylalkane (4a–e) A 3 mmol solution of compound 3a–e in 60 mL of tetrahydrofuran (THF) was treated with 10 mL saturated NaOH solution. The solution was refluxed for 15 h and then allowed to cool to room temperature. The orange solid was collected by filtration, washed successively with ethanol and acetone, and then dried thoroughly under vacuum to obtain the title compounds.

Sodium Salt of 1,4-Bis-[4-[N,N-di-(carboxymethyl)amino]phenoxyl]propane (4a) Yield: 95%, mp >360°C; UV λ<sub>max</sub> (H<sub>2</sub>O) nm (log ε): 316 (4.39); IR (KBr, cm<sup>−1</sup>) ν: 1693 (C=O); 1<sup>H</sup>-NMR (D<sub>2</sub>O, 300 MHz) δ: 6.92 (4H, J=9.3 Hz, ArH), 6.49 (4H, J=9.0Hz, ArH), 4.04 (4H, m, OCH<sub>2</sub>), 3.85 (s, 8H, NCH<sub>2</sub>), 1.76 (4H, CH<sub>2</sub>, 1.55 (2H, CH<sub>2</sub>), 13C-NMR (D<sub>2</sub>O, 500 MHz) δ: 177.0, 146.1, 141.0, 113.6, 109.6, 63.5, 53.2, 25.5; electrospray ionization (ESI)-MS: m/z 489.1 (M−4Na+3H)<sup>+</sup>. Anal. Caled for C<sub>24</sub>H<sub>12</sub>N<sub>4</sub>O<sub>4</sub>: C 59.0, 56.6. Found: C 58.4, 56.4.

Sodium Salt of 1,4-Bis-[4-[N,N-di-(carboxymethyl)amino]phenoxyl]butan (4b) Yield: 91%, mp >360°C; UV λ<sub>max</sub> (H<sub>2</sub>O) nm (log ε): 317 (3.61); IR (KBr, cm<sup>−1</sup>) ν: 1620 (C=O); 1<sup>H</sup>-NMR (D<sub>2</sub>O, 300 MHz) δ: 6.92 (4H, J=9.3Hz, ArH), 6.49 (4H, J=9.0Hz, ArH), 4.04 (4H, m, OCH<sub>2</sub>), 3.85 (s, 8H, NCH<sub>2</sub>), 1.86 (4H, CH<sub>2</sub>), 12C-NMR (D<sub>2</sub>O, 500 MHz) δ: 177.0, 146.1, 141.0, 113.6, 109.6, 66.5, 53.2, 22.3, 20.3; ESI-MS: m/z 503.5 (M−4Na+3H)<sup>+</sup>. Anal. Caled for C<sub>24</sub>H<sub>12</sub>N<sub>4</sub>O<sub>4</sub>: C 48.66, H 3.61, N 4.64.

Sodium Salt of 1,4-Bis-[4-[N,N-di-(carboxymethyl)amino]phenoxyl]pentan (4c) Yield: 93%, mp >360°C; UV λ<sub>max</sub> (H<sub>2</sub>O) nm (log ε): 316 (3.75); IR (KBr, cm<sup>−1</sup>) ν: 1579 (C=O); 1<sup>H</sup>-NMR (D<sub>2</sub>O, 300 MHz) δ: 6.91 (4H, J=9.0Hz, ArH), 6.48 (4H, J=9.3Hz, ArH), 4.0 (4H, m, OCH<sub>2</sub>), 3.85 (s, 8H, NCH<sub>2</sub>), 1.76 (4H, CH<sub>2</sub>), 1.55 (2H, CH<sub>2</sub>), 13C-NMR (D<sub>2</sub>O, 500 MHz) δ: 177.0, 146.1, 141.0, 113.6, 109.6, 66.5, 53.2, 22.3, 20.3; ESI-MS: m/z 517.2 (M−4Na+3H)<sup>+</sup>. Anal. Caled for C<sub>24</sub>H<sub>12</sub>N<sub>4</sub>O<sub>4</sub>: C 49.51, H 4.32, N 4.62. Found: C 49.62, H 4.45, N 4.76.

Sodium Salt of 1,6-Bis-[4-[N,N-di-(carboxymethyl)amino]phenoxyl]hexan (4d) Yield: 93%, mp >360°C; UV λ<sub>max</sub> (H<sub>2</sub>O) nm (log ε): 316 (3.75); IR (KBr, cm<sup>−1</sup>) ν: 1579 (C=O); 1<sup>H</sup>-NMR (D<sub>2</sub>O, 300 MHz) δ: 6.93 (4H, J=9.0Hz, ArH), 6.52 (4H, J=9.3Hz, ArH), 4.01 (4H, m, OCH<sub>2</sub>), 3.87 (s, 8H, NCH<sub>2</sub>), 1.75 (4H, CH<sub>2</sub>), 1.49 (4H, CH<sub>2</sub>), 13C-NMR (D<sub>2</sub>O, 500 MHz) δ: 177.0, 146.2, 141.0, 113.6, 109.7, 66.9, 53.2, 25.5, 22.0; ESI-MS: m/z 531.0 (M−4Na+3H)<sup>+</sup>. Anal. Caled for C<sub>24</sub>H<sub>12</sub>N<sub>4</sub>O<sub>4</sub>: C 50.52, H 4.55, N 4.51. Found: C 50.52, H 4.75, N 4.71.

Sodium Salt of 1,8-Bis-[4-[N,N-di-(carboxymethyl)amino]phenoxyl]octane (4e) Yield: 84%, mp >360°C; UV λ<sub>max</sub> (H<sub>2</sub>O) nm (log ε): 316.5 (4.67); IR (KBr, cm<sup>−1</sup>) ν: 1595 (C=O); 1<sup>H</sup>-NMR (D<sub>2</sub>O, 300 MHz) δ: 6.87 (4H, J=9.0Hz, ArH), 6.59 (4H, J=9.0Hz, ArH), 3.96 (4H, J=6.6Hz, OCH<sub>2</sub>), 3.82 (s, 8H, NCH<sub>2</sub>), 1.68 (4H, CH<sub>2</sub>), 1.34 (4H, CH<sub>2</sub>), 13C-NMR (D<sub>2</sub>O, 500 MHz) δ: 179.9, 149.2, 143.9, 116.4, 112.6, 69.9, 56.1, 28.4, 25.1; ESI-MS: m/z 559.2 (M−4Na+3H)<sup>+</sup>. Anal. Caled for C<sub>24</sub>H<sub>12</sub>N<sub>4</sub>O<sub>4</sub>: C 51.86, H 4.97, N 4.32. Found: C 51.62, H 4.77, N 4.31.

Cell Cultures Primary HDF were purchased from Cascade Biologies (Portland, OR, U.S.A.) and cultured in medium 10<sup>6</sup> supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL of streptomycin. The cells at passages 4–10 were used for this study. The cells were seeded at 1×10<sup>5</sup> cells in 10-cm culture dishes for 24 h before treatment with compounds for the indicated time periods. Control cultures were maintained in media supplemented with...
vehicle (dimethyl sulfoxide (DMSO), 0.1%). No growth or differentiation effects of DMSO were observed under these culture conditions.

**Cell Viability Assays** Cell viability was assessed with CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) by combining WST [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium] and 1-methoxy PMS [1-methoxy-phenazine methosulfate], as described by the manufacturer. Briefly, cells were seeded in 96-well plates at the density of 1×10⁵ cells/well 24 h prior to treatment. Compounds were added to the media as a concentrated stock and the control cells were fed with media containing the same amount of drug-free vehicle. After incubation for 48 h, 10 µL of CCK-8 was added, and cells were further incubated for 3 h. Relative cell viability was obtained by measuring absorbances at 450 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, U.S.A.).

**UV Irradiation and Zymography** To characterize the effect of compounds 4a–e on MMP-1 activity and expression levels in human cells, HDF cells were first treated with or without 10 µM of 4a–e for 24 h. For UV irradiation, the cells were irradiated with 5 mJ/cm² of UV-A (340 nm) for HDF cells, using a UV light irradiator (UVtec Unlimited, Cambridge, England) through a colorless and very thin layer of culture medium. The cells were then washed and incubated with serum-free media for 24 h before they were harvested for analysis. The enzymatic activity of MMP-1 in conditioned media of UVA-irradiated HDF-1 cells was assayed by casein-zymography as previously described. The conditioned media were separated by electrophoresis on an 8.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.33 µg/mL casein. The electrophoresed gel was washed twice with washing buffer containing 2.5% Triton X-100, followed by a brief rinse in washing buffer without Triton X-100. The gel was then immersed for 18 h in incubation buffer of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, and 0.2% Brij 35 at 37°C. After incubation, the gel was stained with 0.25% Coomassie blue R-250. The presence of MMP-1 was identified with a clear band of casein digestion. The protein concentrations of the samples were measured using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer’s protocol (Pierce, Rockford, IL, U.S.A.).

**Western Blot Analysis** Western blot analysis was carried out in HDF cells after treatment with the tested compounds and UVA irradiation. The cells were then washed with ice-cold PBS and lysed in 0.2 mL of lysis buffer (1% NP-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris–HCl, pH 7.6, 10 mM EDTA, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 10 mM NaF, 10 mM β-glycerophosphate, 10 µg/mL protease inhibitor, and phosphatase inhibitor cocktails) for 30 min at 4°C. Equal amounts of protein from each sample were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and transblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). Immunoblotting was performed using antibodies against MMP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), phosphorylated and total JNK, p38, ERK1/2 (Cell Signaling Tech, Danvers, MA, U.S.A.), and β-actin (Chemicon, Temecula, CA, U.S.A.). Signals were visualized with an enhanced chemiluminescence kit (ECL, Amersham, U.K.) followed by exposure to X-ray films.

**Transfection and Luciferase Assays** Human HDF cells were cultured in 60-mm dishes at a seeding density of 3×10⁴ cells/dish. After 24 h, 3 µg of MMP-1 promoter-luciferase reporter and 1 µg of internal reference plasmid pCMVβ were transfected using the cationic polymer transfection reagent jetPEI™ (Qbiogene, Inc., CA, U.S.A.) following the manufacturer’s protocol. The cells were irradiated with or without UVA in the presence or absence of compounds 4a–e for 24 h. The cells were harvested and cell lysates were prepared in 100 µL of 25 mM Tris–Cl, pH 7.6, and 1 mM EDTA. The supernatant was assayed for luciferase activity and β-galactosidase activity. Luciferase activity is expressed as a relative luminescence unit (RLU)/µg protein.

**Statistical Analysis** Student’s t-test for the significance of the difference between the mean values of two independent samples was used. All p values were considered statistically significant at p<0.05. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 11.0 for Windows software.

**Acknowledgments** Financial support from the National Science Council (NSC 100-2632-B-016–007-MY3 to TCC and NSC-100-2632-B-157-001-MY3 to LYH) and the Ministry of National Defense (MAB101-48 and MAB102-9 to TCC) of the Republic of China are gratefully acknowledged.

**References**

1. Epstein J. H., *J. Am. Acad. Dermatol.*, 9, 487–502 (1983).
2. Jenkins G., *Mech. Ageing Dev.*, 123, 801–810 (2002).
3. Gilchrist B. A., *J. Am. Acad. Dermatol.*, 21, 610–613 (1989).
4. Nagase H., Woessner J. F. Jr., *J. Biol. Chem.*, 274, 21491–21494 (1999).
5. Herrmann G., Wlaschek M., Lange T. S., Goerz G., Scharffetter-Kochanek K., *Exp. Dermatol.*, 2, 92–97 (1993).
6. Fisher G. J., Wang Z. Q., Datta S. C., Varani J., Kang S., Voorhees J. J., *N. Engl. J. Med.*, 337, 1419–1428 (1997).
7. Gupta A., Kaur C. D., Jangdey M., Saraf S., *Ageing Res. Rev.*, 13, 65–74 (2014).
8. Pourzand C., Watkin R. D., Brown J. E., Tyrrell R. M., *Proc. Natl. Acad. Sci. U.S.A.*, 96, 6751–6756 (1999).
9. Matsumura Y., Ananthaswamy H. N., *Expert Rev. Mol. Med.*, 4, 1–22 (2002).
10. Brown J. E., Khodr H., Hider R. C., Rice-Evans C. A., *Biochem. J.*, 330, 1173–1178 (1998).
11. Hubaud J.-C., Bombarda I., Decome L., Wallet J.-C., Gaydou E. M., *J. Photochem. Photobiol. B.*, 92, 103–109 (2008).
12. Pygmalion M. J., Ruiz L., Popovic E., Gizard J., Portes P., Marat X., *Lucet-Levannier K., Muller B., Galey J. B., Free Radic. Biol. Med.*, 49, 1629–1637 (2010).
13. Zhang L., Duan D., Cui X., Sun J., Fang J., *Tetrahedron*, 69, 15–21 (2013).
14. Born T., Kontoghiorghe C. N., Spyrou A., Kolnagou A., Kontoghiorghe G. J., *Toxicol. Mech. Methods.*, 23, 11–17 (2013).
15. Dissemend J., Schneider L. A., Brenneisen P., Briviba K., Wenk J., Wlaschek M., Scharffetter-Kochanek K., *Br. J. Dermatol.*, 149, 341–349 (2003).
16. Jurskiewicz B. A., Buettner G. R., *Photochem. Photobiol.*, 59, 1–4 (1994).
17. Kłeszyńska H., Sarapuk J., Oświecimcka M., Witek S., Przestalski S., *Pol. J. Environ. Stud.*, 9, 475–478 (2000).
18. Verstraeten S. V., Oteiza P. J., Fraga C. G., *Biol. Res.*, 37, 293–300 (2004).
19. Varani J., Warner R. L., Gharace-Kermani M., Phantom S. H., Kang S.,
Chung J. H., Wang Z. Q., Datta S. C., Fisher G. J., Voorhees J. J., *J. Invest. Dermatol.*, **114**, 480–486 (2000).

20) Vayalil P. K., Mittal A., Hara Y., Elmets C. A., Katiyar S. K., *J. Invest. Dermatol.*, **122**, 1480–1487 (2004).

21) López-Camarillo C., Ocampo E. A., López Casamichana M. L., Pérez-Plasencia C., Álvarez-Sánchez E., Marchat L. A., *Int. J. Mol. Sci.*, **13**, 142–172 (2012).

22) Rittie L., Fisher G. J., *Aging Res. Rev.*, **1**, 705–720 (2002).

23) Le Panse R., Dubertret L., Coulomb B., *Photochem. Photobiol.*, **78**, 168–174 (2003).

24) Yu Y., Richardson D. R., *J. Biol. Chem.*, **286**, 15413–15427 (2011).

25) Chiang C.-C., Chang T.-C., Tsai H.-J., Hsu L. Y., *Chem. Pharm. Bull.*, **56**, 369–373 (2008).