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# Two sesquiterpene aminoquinones protect against oxidative injury in HaCaT keratinocytes via activation of AMPKα/ERK-Nrf2/ARE/HO-1 signaling

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ABSTRACT

*Aims:* To investigate the cytoprotective effects of two sesquiterpene aminoquinones isolated from the marine sponge *Dysidea fragilis*, Dysidaminone H (DA8) and 3′-methylamino-avarone (DA14), we examined their effects against hydrogen peroxide ($H_2O_2$)-induced oxidative injury in human keratinocyte cell line and elucidated the underlying mechanisms.

*Main methods:* Cell viability was detected using a CCK-8 assay kit. Intracellular reactive oxygen species (ROS) production was measured by fluorescence of 2, 7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA). Messenger RNA and protein expression were measured by real-time quantitative PCR and western blotting analysis. Immunocytochemistry was performed to determine the intracellular location of nuclear factorerythroid 2 p45 related factor 2 (Nrf2). The antioxidant response element (ARE)-luciferase reporter gene assay and RNA interference were used to establish the role of ARE and Nrf2.

*Key findings:* DA8 and DA14 (DAs) resisted $H_2O_2$induced decline of cell viability by inhibiting the accumulation of ROS. Meanwhile, DAs increased HO-1 expression and ARE activity and induced Nrf2 expression, as well as the accumulation of Nrf2 in the cell nucleus. However, silencing of Nrf2 abolished DAs-induced HO-1 expression and ARE luciferase activation. In addition, DAs induced the phosphorylation of both cyclic AMP-activated protein kinase-α (AMPKα) and extracellular signal-regulated kinase (ERK), while specific inhibitors of AMPKα and ERK abrogated HO1 upregulation and Nrf2 activation.

*Significance:* DAs provided cytoprotective effects against $H_2O_2$-induced cytotoxicity by activation of the Nrf2/ARE/HO-1 pathway via phosphorylation of AMPKα and ERK. The findings suggested that DA8 and DA14 might be the candidate therapeutic agents for skin diseases caused by oxidative injury.

## 1. Introduction

A large group of sesquiterpene quinones were isolated from the marine sponge family, *Dysidea,* which were confirmed to possess various bioactive functions including antioxidant, anti-inflammatory effects. For example, a new sesquiterpene quinone was isolated from *Dysidea cf. cristagalli* that showed great inhibition of superoxide production in experiments on human neutrophil cells [1]. Similarly, another sesquiterpene quinone was found to have strong inhibitory action against superoxide production of rat peritoneal leukocytes even when stimulated by *N*-formyl-methionine-leucine-phenylalanine (fMLP) or 12-O-tetradecanoylphorbol-13-acetate (TPA) [2]. Recently, our laboratory isolated a series of sesquiterpene quinones from the South China Sea sponge *Dysidea fragilis* [3–6]. One of them, 3′-methylamino-avarone (DA14), was previously reported to possess antioxidant effects through inhibiting ROS generation in TPA-stimulated human neutrophils [7,8]. But the cytoprotective effects of these sesquiterpene quinones on skin epithelial cells and its underlying molecular mechanisms are unknown.

Human skin plays its role as a barrier to the external environment, but this means its keratinocytes are highly susceptible to oxidative injury that may cause ROS production and further induce skin pathological states even serious diseases, such as skin aging, psoriasis, skin cancer, etc [9–11]. Meanwhile, our bodies have developed several defense mechanisms to maintain ROS at low physiological levels and then to counteract this oxidative stress. Nrf2/HO-1 signaling pathway is an

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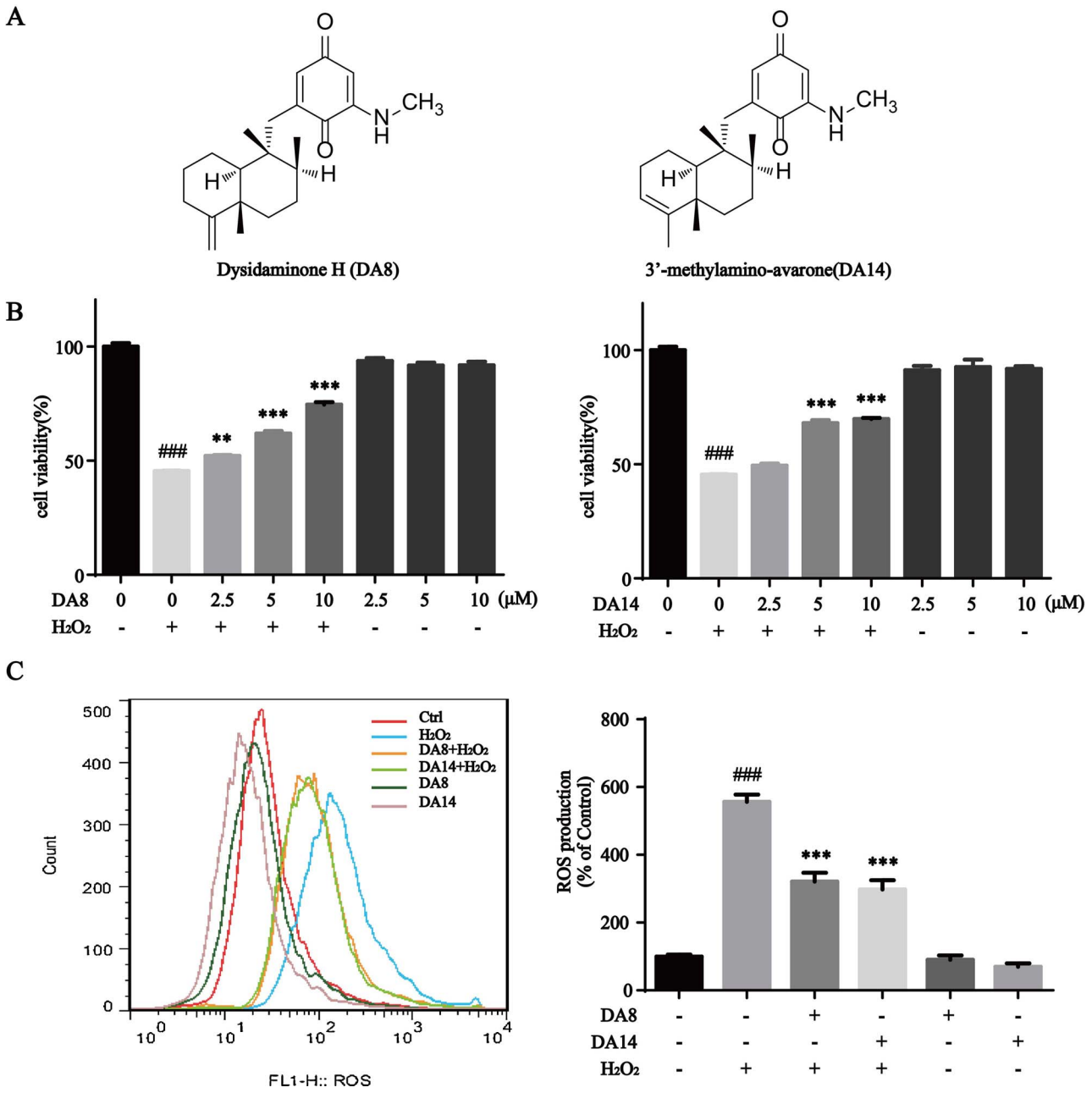


**Fig. 1.** Effects of DAs on cell viability and ROS production in $H_2O_2$-treated HaCaT cells. (A) Chemical structures of DAs (B) Cells were pretreated with DA8 or DA14 (2.5, 5, 10 μM) respectively, for 24 h alone or in the presence or absence of 600 μM $H_2O_2$ for additional two hours. Data represent the mean ± SD of three independent experiments. Significant differences are indicated by ### $p < 0.001$ versus vehicle control, ** $p < 0.01$ and *** $p < 0.001$ compared with $H_2O_2$-treated group. (C) HaCaT cells were treated with the indicated DAs alone for 24 h or in the presence or absence of 100 μM $H_2O_2$ for 2 h, loaded with 10 μM DCFH-DA and analyzed immediately by flow cytometer. Significant differences are indicated by ### $p < 0.001$ versus vehicle control, *** $p < 0.001$ compared with $H_2O_2$-treated group.

important mediator of cellular injury in response to oxidative stress [12–14]. Hseu YC et al. indicated that ergothioneine exhibits dermatoprotective effects against UVA injury by induction of Nrf2/ARE-mediated antioxidant genes in human keratinocytes [15]. Zhao R et al. found that silencing of Nrf2 significantly reduced the expression of many antioxidant enzymes including HO-1 and sensitized the cells to acute cytotoxicity in HaCaT cells [16].

In the present study, we explored the effects of both DA14 and its structural analog Dysidaminone H (DA8) on $H_2O_2$-induced oxidative injury in HaCaT cells. We determined that DAs protected against oxidative injury in HaCaT cells via activation of Nrf2/ARE/HO-1 signaling mediated by phosphorylation of AMPKα/ERK, which provided a theoretical evidence for the discovery of new antioxidant compounds or drug candidates in treatment of skin diseases associated with oxidative stress.

## 2. Materials and methods

### 2.1. Materials

Dysidaminone H (DA8) and 3′-methylamino-avarone (DA14) were isolated from the marine sponge *Dysidea fragilis* in our laboratory, with a purity of 98% (Supplementary methods 1). The compounds (20 mM) stock solutions were dissolved in dimethyl sulfoxide (DMSO) reagent and stored at −20 °C until use. Dulbecco's modified Eagle medium (DMEM), Opti-MEM® I, Fetal bovine serum (FBS), 0.25% Trypsin-EDTA, Penicillin-Streptomycin were from Gibco Life technologies (Temecula, USA). CCK-8 assay kit was purchased from Dojindo

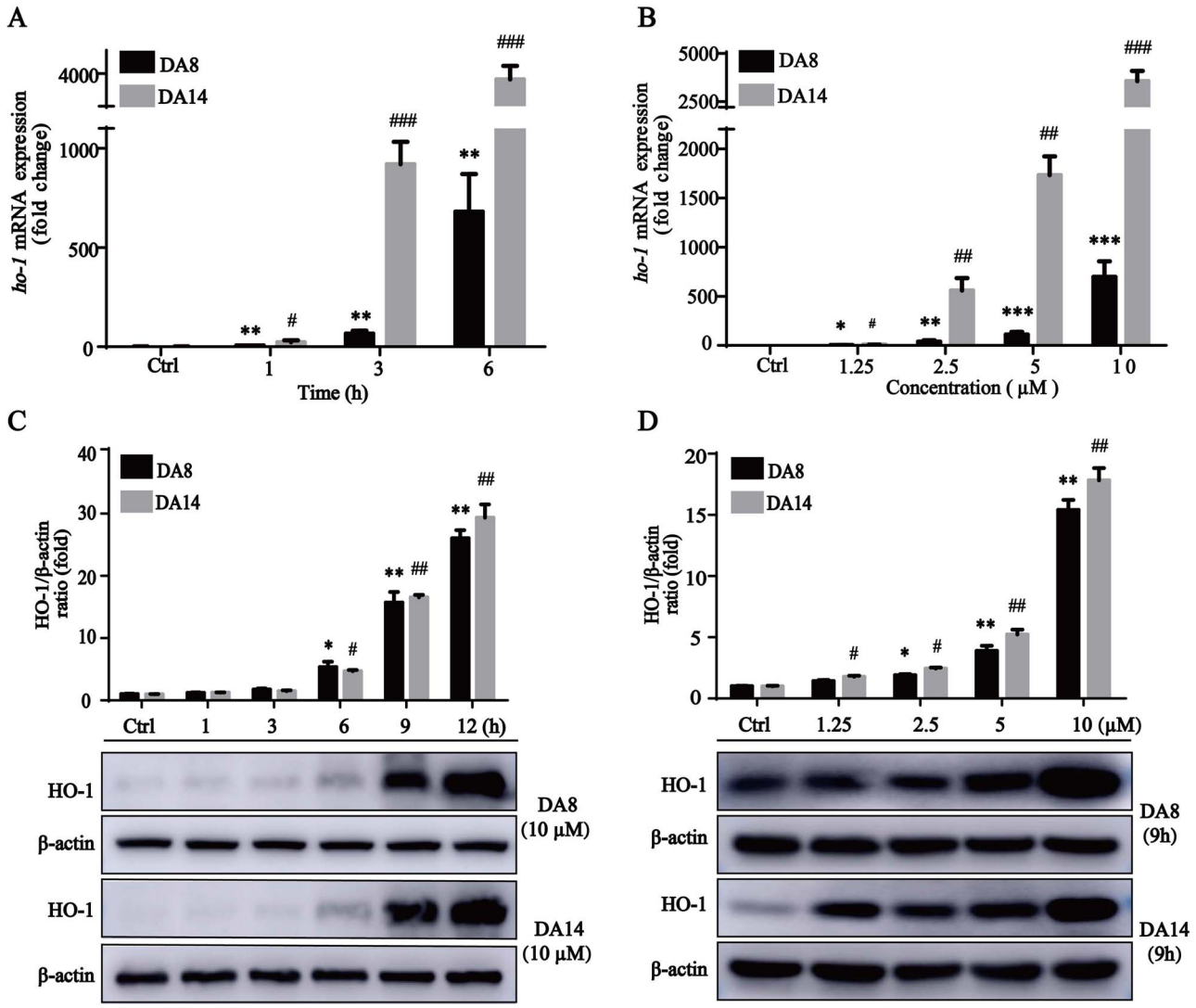


**Fig. 2.** Effects of DAs on HO-1 expression in HaCaT cells. Cells were treated with 10 μM DA8 or DA14 for indicted time periods (A, C), or treated for 9 h with indicted concentrations (B, D). β-Actin was used as a loading control. Values are presented as mean ± SD (n = 3). All data are from three separate experiments. Significant differences are indicated by $*p < 0.05$, $** p < 0.01$ or $*** p < 0.001$ versus Ctrl-DA8, $\# p < 0.05$, $\#\# p < 0.01$ or $\#\#\# p < 0.001$ versus Ctrl-DA14.

Laboratories (Tokyo, Japan). Monoclonal antibody of β-actin, Nrf2, p-ERK1/2, p-p38, p-JNK, p-AMPKα were from Cell Signaling Technology (Danvers, USA). GAPDH monoclonal antibody, LAMN polyclonal antibody and HO-1 polyclonal antibody were from ABclonal Biotechnology Co (Wuhan, China). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). 4′, 6′-Diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, USA). The human special Nrf2 siRNA (sense 5′- CCAGUUGACAGUGAACUCATT -3′; antisense 5′- UGAGUUCACUGUCAACUGGTT-3′), negative control duplexes were obtained from GenePharma (Shanghai, China). The MEK inhibitor U0126 and AMPK inhibitor Compound C were supplied by MedChemExpress (NJ, USA). The HO-1 inhibitor Zinc protoporphyrin-9 (ZnPPIX) was purchased from Thermo Fisher (MA, USA).

### 2.2. Cell culture and treatment

A spontaneously immortalized skin keratinocyte cell line HaCaT, was obtained from iCell Bioscience Inc. (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a humidified 5% $CO_2$ and 95% air incubator. Cells were subcultured or seeded at 80–90% confluence. Unless otherwise stated, seeding density was $3 \times 10^5$ cells/mL. During all the experiments, the amount of DMSO in cell culture medium was controlled at less than 0.1% (v/v). All experiments were performed independently in triplicate.

### 2.3. Cell viability assay and intracellular ROS measurement

The effects of DAs on cell viability were determined using CCK-8 assay. Briefly, the cells were seeded in 96 well plates at a density of $2 \times 10^4$ cells/well, and then incubated at 37 °C for 24 h. After being pretreated with different concentrations of DAs for 24 h alone or in the presence or absence of 600 μM $H_2O_2$ for an additional two hours, cells were incubated with the CCK-8 solution for one-hour. The absorbance at 450 nm was measured in a full wavelength microplate reader (spectra MAX190, Molecular Devices, USA). Each group was established in five wells and performed in triplicate independently. Intracellular ROS levels were measured using a reactive oxygen species assay kit by flow cytometer. After incubation with DAs alone or in the presence or absence of $H_2O_2$, cells were incubated with DCFH-DA (10 μM) probe at 37 °C for 30 min and washed twice with PBS. Then DCF fluorescence intensities were measured at excitation and emission

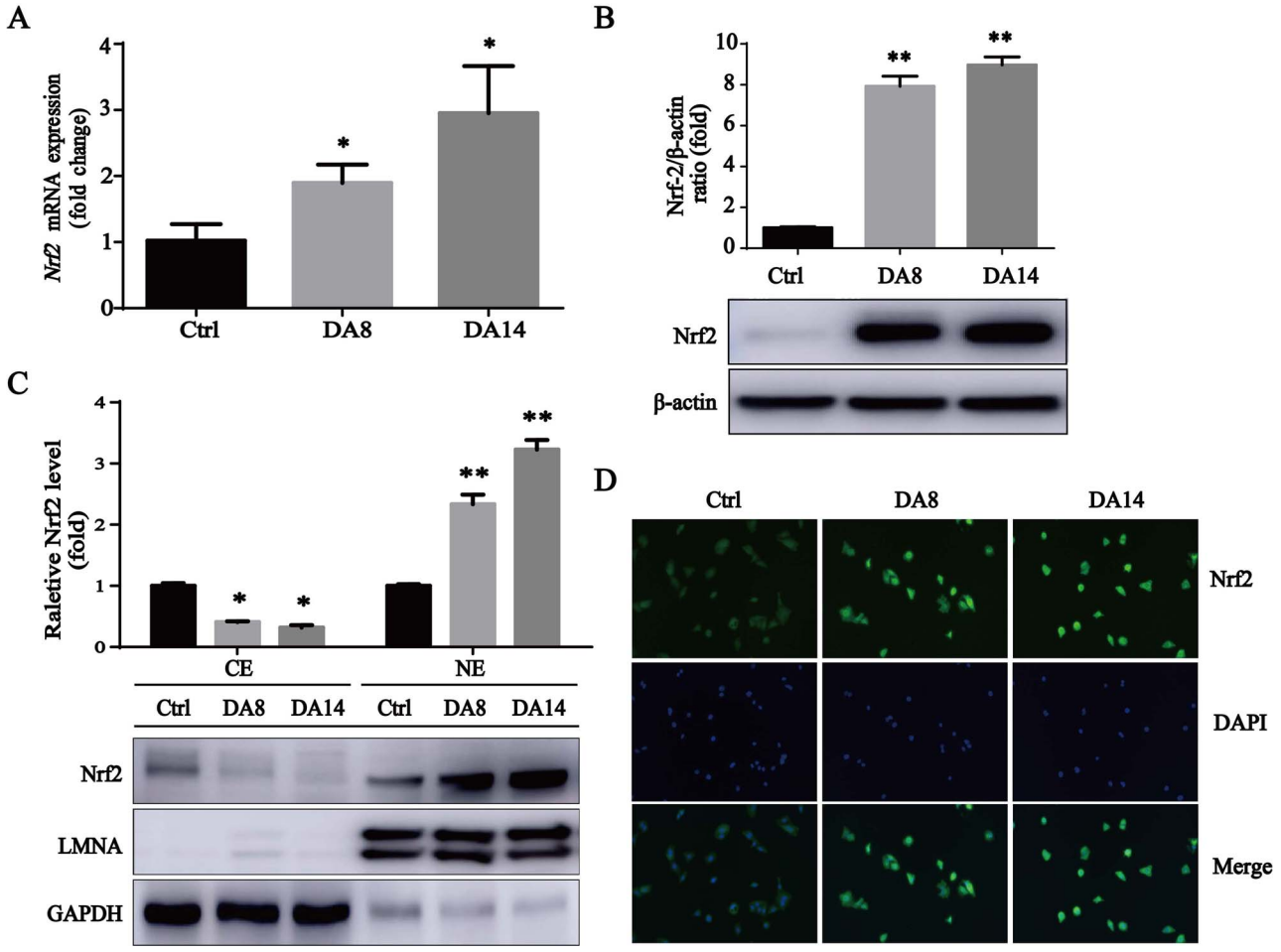


**Fig. 3.** DAs induced the expression and nuclear translocation of Nrf2. Cells were treated with 10 μM DA8 or DA14 for 6 h (A–D). RT-qPCR was used to assess the *Nrf2* mRNA transcript levels (A) and western blotting analysis was performed to detect Nrf2 protein expression (B). Values are presented as mean ± SD (n = 3). Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ versus control group. (C) Cytoplasmic and nuclear fractionation kit was used to obtain the cytosolic and nuclear protein. LMNA and GAPDH were considered as nuclear and cytosolic loading control, respectively. And bands were quantified. CE, Cytosolic extract. NE, nuclear extract. Values are presented as mean ± SD (n = 3). Significant differences are indicated by * $p < 0.05$ versus Ctrl-CE group and ** $p < 0.01$ versus Ctrl-NE group. (D) Immunocytochemistry analysis was performed to determine the nuclear localization of Nrf2. Cellular morphologies were visualized with a fluorescence microscope (200×). The representative images of three independent experiments are shown here.

wavelengths of 485 and 535 nm, separately.

### 2.4. Total RNA isolation and real-time quantitative PCR

Total cellular RNA was extracted from HaCaT cells using RNA simple Total RNA Kit, provided by TIANGEN BIOTECH (Beijing, China). Total RNA was reverse-transcribed to cDNA using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Japan), and PCR amplification was quantified by SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) using LightCycler 480 System (Roche, Switzerland). Primers for human genes were synthesized by Sangon Biotech (Shanghai, China) and the sequences of primer pairs were as follows: *ho-1*, forward (5′-AAGAGGCTAAGACCGCCTTC) and reverse (5′- GCATAAATTCCCACTG CCAC); *Nrf2*,forward (5′- TCAGCGACGGAAAGAGTATGA) and reverse (5′-CCACTGGTTTCTGACTGGATGT); *β-actin*, forward (5′-CCTGGCACC CAGCACAAT) and reverse (5′-GGGCCGGACTCGTCATAC). The amount of each gene was determined relative to *β-actin* gene.

### 2.5. Total protein preparation and western blotting analysis

Cells were harvested and lysed with RIPA buffer (containing 1% PMSF). Protein concentration was determined by using Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China). The whole-cell lysates were separated by SDS-PAGE (10%). After electrophoresis, proteins were transferred onto polyvinyl-difluoride (PVDF) membrane (0.2 μm), then blocked with blocking buffer for 1.5 h. The membranes were incubated with primary antibody at the appropriate concentration overnight at 4 °C, followed by secondary antibodies (1:5000) for one hour at room temperature. For each step, the membranes were washed with 1 × TBST buffer five times. Finally, the protein bands were detected by NcmECL Ultra Luminol/Enhancer Reagent (A) and NcmECL Ultra Stabilized/Peroxide Reagent (B) (1:1, New Cell & Molecular Biotech, China) and profiled by Amersham Imager 600 gel imaging system (GE Healthcare, USA). Image J software was used to perform the gray value analysis.

### 2.6. Cytosolic and nuclear protein extraction

The HaCaT cells were seeded in 6-well plates and permitted to adhere for 24 h in the incubator, before being treated with DAs (10 μM) for an additional 6 h. The extraction of cytosolic and nuclear proteins was performed according to the instruction of Minute™ cytoplasmic and nuclear fractionation kit (Invent Biotechnologies, USA). Cells were lysed using cytoplasmic extraction buffer for 5 min then centrifuged for 5 min at 14 000 rpm at 4 °C. The supernatant was used as the cytosolic proteins. The pellets were washed twice with cold PBS, added with nuclear extraction buffer, vortexed vigorously for 15 s, and incubated on ice for one minute. After repeating four times for 15 s of vortexing

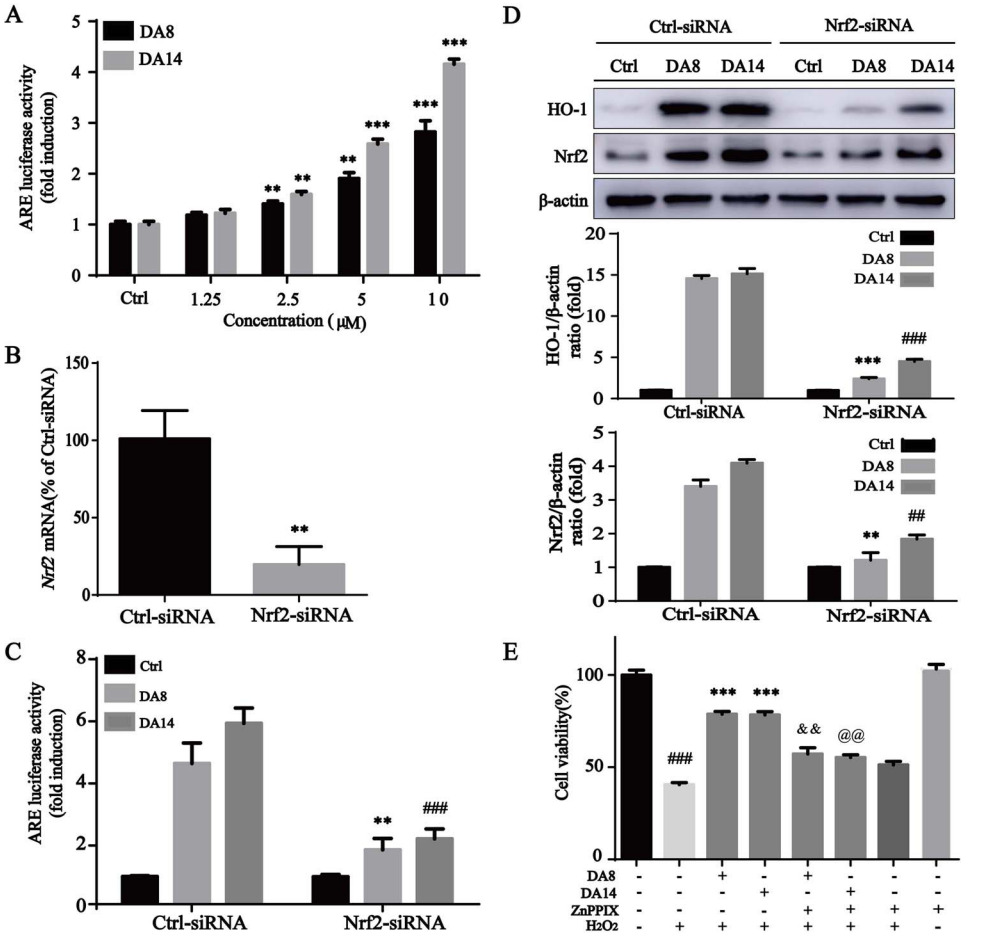


**Fig. 4.** DAs-induced ARE activation and HO-1 expression were abolished by Nrf2 siRNA. (A) DA8 or DA14 concentration-dependently induced the ARE-luciferase activity. Values are presented as mean ± SD (n = 3). Significant differences are indicated by $^{**}p < 0.01$ or $^{***}p < 0.001$ versus control group. (B) HaCaT cells were transfected with control siRNA and Nrf2 siRNA for 6 h. Significant differences are indicated by $^{**}p < 0.01$ versus Ctrl-siRNA group. (C) Cells were transiently transfected with either Nrf2 siRNA or control siRNA as described above along with either the ARE luciferase construct or control vector. The ARE luciferase activity was measured. Significant differences are indicated by $^{**}p < 0.01$ versus Ctrl-siRNA-DA8, ### $p < 0.001$ versus Ctrl-siRNA-DA14. (D) HaCaT cells were transfected with control siRNA or Nrf2 siRNA. Cells were treated with 10 μM DAs for 6 h. Cells were lysed and the proteins expression of HO-1 and Nrf2 were analyzed using western blotting assay. Statistical significance shown as $^{**}p < 0.01$ or $^{***}p < 0.001$ versus Ctrl-siRNA-DA8, ## $p < 0.01$ or ### $p < 0.001$ versus Ctrl-siRNA-DA14. (E) Cells were pretreated with 10 μM ZnPPIX for 1 h, and incubated with DA8 or DA14 (10 μM) for 24 h. Then cells were stimulated with 600 μM $H_2O_2$ for 2 h. Data represent the mean ± SD (n = 3). Significant differences are indicated by ### $p < 0.001$ versus vehicle control, $^{***}p < 0.001$ compared with $H_2O_2$-treated group, && $p < 0.01$ versus DA8 + $H_2O_2$ group, @@ $p < 0.01$ compared with DA14 + $H_2O_2$ group.

and one-minute incubation intervals, cell lysates were centrifuged at 14 000 rpm at 4 °C for 30 s. The supernatant was used as the nuclear proteins.

### 2.7. Immunocytochemistry of Nrf2

Immunocytochemistry was performed to examine the nuclear translocation of Nrf2. Cells were seeded in 24 well plates at a density of $2 \times 10^4$ cells per well and fixed with 4% paraformaldehyde (preheating at 37 °C) for 10 min, then permeabilized in 0.4% Triton X-100 for 15 min. After that, cells were blocked with 1% bovine serum albumin (BSA) for 1 h at 37 °C, followed by incubation with diluted (1:100) Nrf2 primary antibodies overnight at 4 °C and then with corresponding diluted (1:1000) goat anti-rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Washed with PBS, samples were stained by DAPI (100 ng/mL) solution for 10 min at 37 °C. Finally, the cells were visualized by a fluorescence microscope (Nikon, Japan).

### 2.8. The ARE-luciferase reporter gene assay

Cells ($2 \times 10^4$) were seeded into 24-well plates prior to transfection. Cells were transiently cotransfected with Firefly luciferase reporter plasmid harboring the ARE-promoter plasmid p-ARE-Luc and Renilla luciferase plasmid p-RL using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After 24 h transfection, cells were treated with DA8 or DA14 for additional 6 h and then washed twice with PBS and lysed with 1 × reporter lysis buffer. The supernatant was used for the luciferase assay. The Firefly luciferase or Renilla luciferase assay reagent was added, and the luciferase activity was measured by the luminometer. The Rellina values were used for normalizing the luciferase activity and the results were expressed as fold transactivation.

### 2.9. RNA interference

HaCaT cells were seeded in 6-well plates at the density of $1.0 \times 10^5$ cell/mL. Nrf2 siRNA and negative control siRNA were transfected using Lipofectamine 2000 according to the kit descriptions. The cells were incubated for 6 h and then provided with fresh medium containing 10% FBS. Total mRNA was extracted for real-time quantitative PCR to certify Nrf2 gene has been knockdown. Transfected cells were treated with DAs for 6 h, western blotting was used to test protein expression.

### 2.10. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis of significances was carried out by one-way analysis of variation (ANOVA) followed by *t*-tests using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). A probability value of $p < 0.05$ was considered as the criterion for statistical significance.

## 3. Results

### 3.1. Protective effects of DAs against $H_2O_2$-induced cytotoxicity triggered by ROS accumulation

$H_2O_2$ treatment decreased cell viability through oxidative processes in different cell types, including HaCaT cells [17–19]. Therefore, we

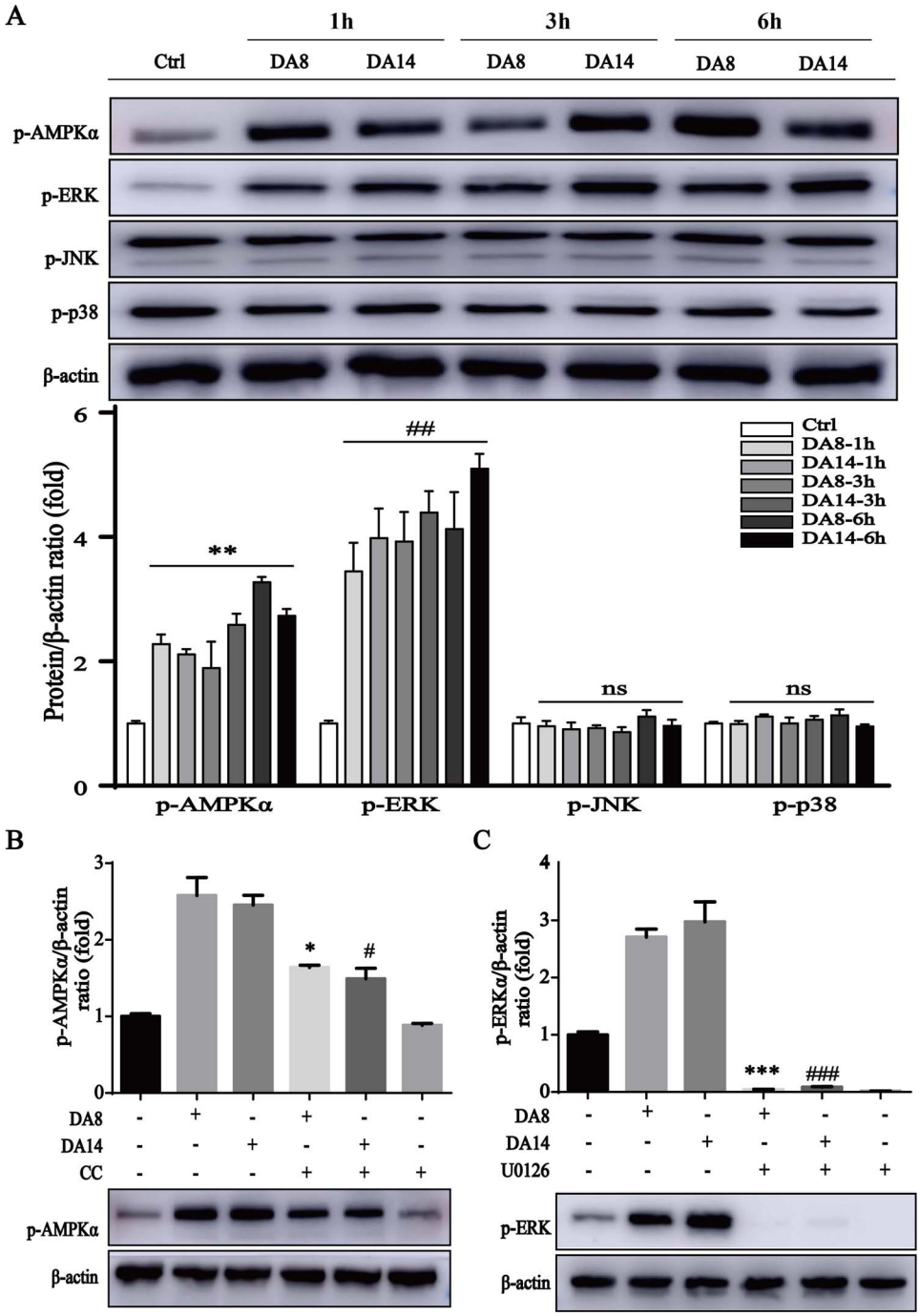


**Fig. 5.** Effects of DAs on the activation of AMPKα, ERK in HaCaT cells. (A) Cells were treated with DAs (10 μM) for indicated time intervals. β-Actin was considered as loading control. Quantitative data for p-AMPKα, p-ERK, p-JNK and p-p38 are shown. Statistical significance shown as $^{**}p < 0.01$ or $\#\# p < 0.01$ versus control group. (B, C) Cells were pre-incubated with Compound C (CC) (10 μM) and U0126 (10 μM), pharmacological inhibitors of AMPKα, ERK, respectively, for 1 h before treatment with DAs. Quantitative data are shown and significant differences are indicated by $^{*} p < 0.05$ or $^{***} p < 0.001$ versus DA8 group and $\# p < 0.5$ or $\#\#\# p < 0.001$ versus DA14 group.

explored whether DAs (Fig. 1A) could exhibit cytoprotective effects on HaCaT cells. The results indicated DAs almost showed no toxicity to HaCaT cells at the indicated concentrations and DAs could counteract $H_2O_2$-induced cell death with concentration-dependent effects (Fig. 1B). To confirm the antioxidant properties of DAs, cells were pretreated with DAs for 24 h, and we found ROS production decreased significantly compared with $H_2O_2$-treated group (Fig. 1C). Therefore, the results suggested that DAs might exert cytoprotective effects by inhibiting the accumulation of ROS.

### 3.2. DAs enhance the expression of HO-1 in HaCaT cells

HO-1 plays a vital role in cytoprotective effects of the cells [20,21]. Therefore, we examined whether HO-1 was involved in DAs activity in HaCaT cells. As shown in Fig. 2A–D, DAs significantly up-regulated HO-1 mRNA and protein expression time- and concentration-dependently in HaCaT cells. The results confirmed that HO-1 was involved in DAs activity of these cells. We also found an evident increase of HO-1 protein expression occurred after 9 h incubation with 10 μM DAs, compared with sulforaphane (SFN), an inducer of HO-1 (Supplementary Fig. 1A). It suggested that DAs were more potent than SFN in HO-1 induction. In

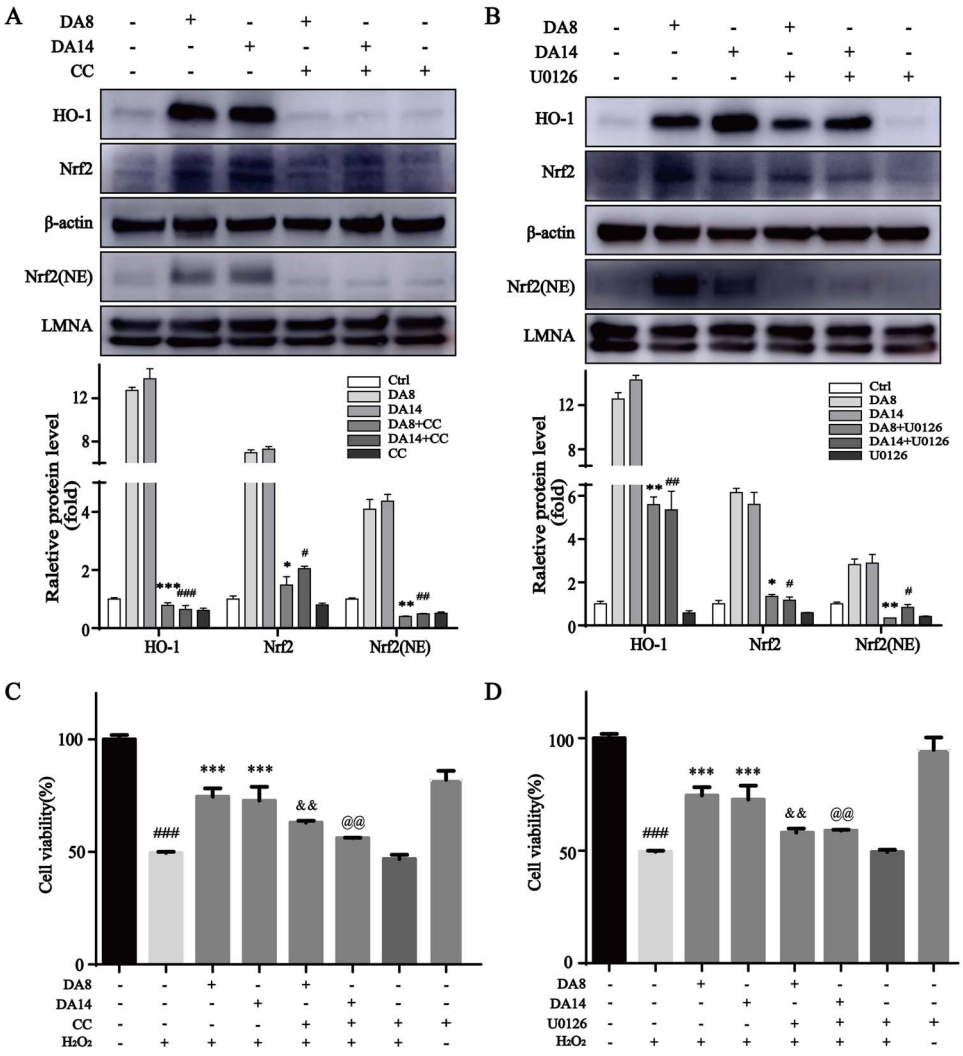


**Fig. 6.** Roles of AMPKα and ERK in DAs-induced activation of Nrf2/HO-1 in HaCaT cells (A. B) Cells were pretreated with 10 μM Compound C (CC) or U0126 for 1 h prior to DA8 or DA14 (10 μM) for 9 h. Statistical significance shown as $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ versus DA8 group, # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$ versus DA14 group. (C. D) Cells were pretreated with 10 μM Compound C or U0126 for 1 h, and incubated with DA8 or DA14 (10 μM) for 24 h. Then cells were stimulated with 600 μM $H_2O_2$ for 2 h. Data represent the mean ± SD of three independent experiments. Significant differences are indicated by ### $p < 0.001$ versus vehicle control, *** $p < 0.001$ compared with $H_2O_2$-treated group, && $p < 0.01$ versus DA8 + $H_2O_2$ group, @@ $p < 0.01$ compared with DA14 + $H_2O_2$ group.

addition, we found when DAs were applied, both the catalase protein expression and glutathione (GSH) levels were increased, as well as the mRNA levels of anti-oxidative genes *nqo1, akr1c1* and *gclc*, but the changes were not as robust as HO-1 induction (Supplementary Fig. 2). In addition, ZnPPIX, an inhibitor of HO-1 enzyme, was used to pretreat cells before DAs incubation. We found the protective effects of DAs against $H_2O_2$ were attenuated by ZnPPIX (Fig. 4E), indicating that DAs-enhanced HO-1 was vital for counteracting with oxidative damage.

### 3.3. DAs up-regulated the expression of Nrf2 and induced its nuclear translocation

In general, activation of Nrf2 is necessary for antioxidant gene expression, therefore we examined if DA8 or DA14 could up-regulate the expression and the nuclear accumulation of Nrf2. We observed that treatment of HaCaT cells with DAs for 6 h resulted in increased Nrf2 mRNA and protein levels, combined with Nrf2 nuclear translocation (Fig. 3A–C). Immunocytochemistry analysis verified the accumulation of Nrf2 in nucleus (Fig. 3D).

### 3.4. DAs-induced HO-1 expression is dependent on Nrf2 activation

Treatment with DAs dose-dependently increased the ARE luciferase activity (Fig. 4A). To confirm the involvement of Nrf2 in DA8 or DA14-induced ARE activation, HaCaT cells were transfected with siRNA against Nrf2. Real-time quantitative PCR analysis demonstrated that the mRNA levels of Nrf2 were largely decreased by Nrf2 siRNA (Fig. 4B). Significant increases in ARE luciferase activity by DA8 or DA14 were also mostly suppressed by silencing Nrf2 (Fig. 4C). On the other hand, silencing of Nrf2 decreased DAs-enhanced HO-1 expression (Fig. 4D), which suggested that DAs-induced expression of HO-1 was dependent on Nrf2 activation in HaCaT cells. Furthermore, as the transcription factor Bach1 is a potent repressor of HO-1 [22], it may be involved in HO-1 induction by DAs, we explored the cytoplasmic and nuclear Bach1 protein levels in HaCaT cells treated with vehicle control and DAs. However, we unexpectedly observed that Bach1 protein levels in DAs-treated HaCaTs did not change compared with in vehicle control cells neither in nucleus nor in cytoplasm (Supplementary Fig. 1B).

### 3.5. DAs induced the phosphorylation of AMPKα and ERK in HaCaT cells

Previous studies have discovered that upstream kinases, such as AMPKα and mitogen-activated protein kinases (MAPKs), mediated the activation of the Nrf2/ARE signaling pathway in various cell types [23,24]. Therefore, we investigated if DAs could induce the activation of ERK, JNK, p38 and AMPKα. Treatment of HaCaT cells with DAs for different time periods and then the activation of all the kinases were analyzed by western blotting. As shown in Fig. 5A, DAs increased the phosphorylation of AMPKα and ERK but not JNK or p38. Furthermore, DAs-induced phosphorylation of AMPKα and ERK were halted by pretreatment of their specific inhibitors, Compound C or U0126, respectively (Fig. 5B–C). The results suggested that DAs activated the AMPKα

and ERK signaling in HaCaT cells.

### 3.6. The AMPKα and ERK signaling pathways contribute to DAs-induced Nrf2 activation and HO-1 up-regulation

In order to explore whether DAs induce Nrf2 activation and HO-1 expression through phosphorylation of AMPKα and ERK, we used the corresponding inhibitors to pretreat HaCaT cells prior to DAs. We found that pharmacological inhibition of AMPKα and ERK attenuated DAs-induced Nrf2 and HO-1 expression, respectively, as well as the nuclear localization of Nrf2 (Fig. 6A–B). In addition, the cytoprotective effects of DAs were suppressed by Compound C or U0126, separately (Fig. 6C–D). Our results suggested that the cytoprotection of DAs was mediated by antioxidant properties via activation of AMPKα/ERK.

## 4. Discussion

This study revealed for the first time, that two sesquiterpene aminoquinones from the marine sponge *Dysidea fragilis*, DA8 and DA14, provided protective action against $H_2O_2$-induced cytotoxicity and oxidative stress through Nrf2/ARE/HO-1 pathway activated by phosphorylation of AMPKα and ERK in human keratinocyte cells.

Previous research verified that DA14 showed no sign of cytotoxicity [8]. However, so far, little is reported about DA8 related activity. To mimic signaling activated by endogenously produced ROS, HaCaT cells were stimulated with $H_2O_2$ to induce oxidative injury. Our data demonstrated that DAs protected cells against $H_2O_2$-induced cytotoxicity through reduction of ROS content.

Since numerous studies revealed that Nrf2/ARE/HO-1 signaling plays an important role in response to oxidative stress and cytoprotection [14,25–27], we supposed the protective effects of DAs might be related to the activation of Nrf2/ARE/HO-1 pathway. HO-1 is a key 32-kDa enzyme involved in the adaptive response to cellular stress, through catalyzing of the rate-limiting step in toxic heme catabolism, leading to the generation of biliverdin, free iron and carbon monoxide (CO). Both bilirubin (the product of biliverdin degradation) and CO may protect cells against oxidative stress [20,28,29]. Additionally, HO-1 deficiency induced abnormal cellular homeostasis and the vulnerability to stressful injury not only in mice model but also in human cell studies [30,31]. In this study, we found that DAs induced the expression of HO-1 in HaCaT cells, at the mRNA and protein levels and DAs were more potent than sulforaphane as inducers of HO-1.

HO-1 is mainly regulated by Nrf2/ARE signal transduction pathway [32]. Nrf2 is a member of the NF-E2 family of the basic leucine zipper transcription factors. Under normal condition, Nrf2 was attached with its negative regulator Keap1 and rapidly degraded through the ubiquitin-26S proteasome pathway in cytoplasm. Once activated, Nrf2 could translocate into the cell nucleus [33], and then bind to the ARE sequences, along with other transcription factors, such as small Maf protein, which accelerate the expression of various cytoprotective genes [34]. Nrf2 plays a key role in the regulation of the ARE-driven cellular defense system against oxidative stress [35]. Therefore, we examined the effects of DAs on Nrf2-ARE signaling, and found that DAs could activate ARE genes concentration-dependently and contribute to both expression and nuclear accumulation of Nrf2. Additionally, the activation of ARE sequences could be countered by Nrf2 siRNA whereas DAs-induced HO-1 expression could be negated by silencing of Nrf2. The findings suggested that DAs-induced ARE activation was mediated by the transcription factor Nrf2 and that HO-1 expression stimulated by DAs was dependent on the Nrf2 activation. The results were similar to the study by Hseu YC et al. who have demonstrated that silencing of Nrf2 in human keratinocytes would decrease the expression of HO-1 [15]. However, there are still increases of both ARE activity and HO-1 expression by DAs although Nrf2 was silenced with Nrf2 siRNA, suggesting that DAs may have promoted HO-1 transcription by other transcriptional factors. Researchers ever reported that transcription factor c-Jun, one important member of activator protein 1 (AP-1) family could bind to ARE and then increased phase II antioxidant enzyme expression, including HO-1 [36–38]. So DAs may be able to induce c-Jun binding to ARE and modulate HO-1 transcription. On the other hand, the inhibition rate of Nrf2 siRNA is about 80%. That is to say, Nrf2 siRNA could not entirely inhibit the Nrf2 expression.

AMPKα is a vital cellular energy sensor by maintaining energy homeostasis in response to metabolic stress such as oxidative stress, inflammation, etc [39]. MAPKs signaling pathways are involved in diverse physiological processes, and also play essential roles in overcoming oxidative stress [40,41]. Several studies linked the AMPKα or MAPKs signaling pathways to the activation of the Nrf2/ARE/HO-1 signal pathway [42–47]. Our results indicated that AMPK and ERK were involved in DAs-induced Nrf2 activation. Pretreatment with the specific inhibitors of p-AMPKα or p-ERK suppressed phase II antioxidant enzyme HO-1 gene expression and Nrf2 activity. The results elucidated that activation of AMPKα and ERK was associated with Nrf2 activation and HO-1 expression in DAs-induced HaCaT cells. Moreover, the cytoprotection of DAs was abolished by the inhibition of AMPKα or ERK phosphorylation. These results suggested that DAs-mediated Nrf2 antioxidant signaling pathways may be regulated by the activation of AMPKα and ERK in HaCaT cells.

## 5. Conclusion

In conclusion, we found two sesquiterpene aminoquinones from *Dysidea fragilis* sponge, DA8 and DA14, induced Nrf2 activation and HO-1 expression via phosphorylation of AMPKα/ERK, and enhanced cellular resistance to $H_2O_2$-induced cytotoxicity by suppressing intracellular ROS formation in HaCaT cells. Therefore, these two sesquiterpene aminoquinones might be the candidate therapeutic agents for skin diseases related to oxidative injury.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.02.034.

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