Tetrandrine Enhances H₂O₂-Induced Apoptotic Cell Death Through Caspase-dependent Pathway in Human Keratinocytes

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Abstract. *Background:* Tetrandrine, a bis-benzylisoquinoline alkaloid, induces apoptosis of many types of human cancer cell. Hydrogen peroxide (H₂O₂) is a reactive oxygen species inducer; however, there are no reports to show whether pre-treatment of tetrandrine with H₂O₂ induces more cell apoptosis than H₂O₂ alone. Thus, the present study investigated the effects of tetrandrine on H₂O₂-induced cell apoptosis of human keratinocytes, HaCaT, in vitro. *Materials and Methods:* HaCaT cells were pre-treated with and without tetrandrine for 1 h, and then treated with H₂O₂ for examining cell morphological changes and cell viability using contrast-phase microscopy and propidium iodide (PI)

exclusion assay, respectively. Cells were measured apoptotic cell death by using annexin V/PI double staining and further analyzed by flow cytometer. Cells were further assessed for DNA condensation using 2-(4-amidinophenyl)-6-indolecarbamidine staining. Western blotting was used to measure expression of apoptosis-associated proteins and confocal laser microscopy was used to measure the protein expression and nuclear translocation from the cytoplasm to nuclei. *Results:* Pre-treatment of tetrandrine for 1 h and treatment with H₂O₂ enhanced H₂O₂-induced cell morphological changes and reduced cell viability, whilst increasing apoptotic cell death and DNA condensation. Furthermore, tetrandrine significantly increased expression of reactive oxygen species-associated proteins such as superoxide dismutase (Cu/Zn) and superoxide dismutase (Mn) but significantly reduced the level of catalase, which was also confirmed by confocal laser microscopy. It also increased expression of DNA repair-associated proteins ataxia telangiectasia mutated, ataxia-telangiectasia and Rad3-related, phospho-P53, P53 and phosphorylated histone H2AX, and of pro-apoptotic proteins BCL2 apoptosis regulator-associated X-protein, caspase-3, caspase-8, caspase-9 and poly ADP ribose polymerase in HaCaT cells. *Conclusion:* These are the first and novel findings showing tetrandrine enhances H₂O₂-induced apoptotic cell death of HaCaT cells and may provide a potent approach for the treatment of proliferated malignant keratinocytes.

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Skin covers the human body's outer surface and is the first line of defense against microbial and chemical attacks (1). The skin is therefore exposed to various stressors (2, 3) such as UV light (4, 5), and air pollutants (6, 7), including heavy metals (Cu, Mn, Ni, Pb, and Ti) and polycyclic aromatic hydrocarbons (8). Prolonged exposure to such stresses may cause skin damage, leading to the induction of skin cancer (9). The majority of the epidermis consists of keratinocytes (10), which form a physical barrier, and they have diverse receptors for the stimulation of signaling transduction pathways to other layers of the skin (11, 12). However, epidermal keratinocytes are very susceptible to the effects of environmental pollutants, leading to oxidative stress, which induces cancer, aging, and inflammatory disorders (13, 14). Abnormalities of keratinocyte growth leads to skin disorders, such as psoriasis, inflammatory allergic diseases, and chronic wounds (15). Reactive oxygen species (ROS), including most free radicals, have been shown to damage cellular proteins, lipids, and DNA (16) which can result in skin disorders (17). Oxidative stress is an important cause of DNA damage (18) and may induce about 10,000 DNA alterations per cell per day (19). In the body, endogenous antioxidant enzymes, including nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone) 1 and heme oxygenase-1, function to reduce ROS in order to maintain normal skin biology (20). ROS at low levels is essential for cells to avoid extracellular invaders and to maintain cellular signaling. Overproduction of ROS by oxidative stress can injure DNA, proteins and lipids (21), resulting in cancer induction, cardiovascular diseases, and neurodegenerative diseases (22). Therefore, approaches for protecting the skin against oxidative stress are needed.

Hydrogen peroxide (H_2O_2) is an unstable ROS, and induces oxidative stress (14). Exogenous H_2O_2 inhibited porcine trophectoderm, reduced cell viability, arrested cells in S and G₂/M phases, and increased cell apoptosis and autophagy (23). Moreover, H_2O_2 was reported to freely pass through the cell membrane to damage cells, such as during replicative senescence (19). H_2O_2 also triggers apoptosis of keratinocytes *via* the release of cytochrome c, cleaved caspase activity, and pro-apoptotic gene expression (21).

Tetrandrine, a bis-benzylisoquinoline alkaloid, was extracted from the dried root of *Stephania tetrandra*. Tetrandrine has anti-allergenic (24) and anti-inflammatory (25) properties. It is clinically used for treating rheumatoid arthritis (26, 27), silicosis (28), and cardiovascular disease (29) in the Chinese population. Furthermore, tetrandrine exhibits anticancer activities *via* induction of apoptosis in human breast (30), gastric (31), lung (32), oral (33), and liver (34) cancer, nasopharyngeal (35) and epidermoid (36) carcinoma cells, and laryngeal cancer stem cells (37). However, the bioactivity of tetrandrine pre-treatment in skin cells subjected to oxidative stress has not yet been studied. Therefore, in the

present study, the effects of tetrandrine on apoptosis of H_2O_2 -treated HaCaT human keratinocytes were evaluated *in vitro*. Furthermore, the molecular mechanisms of apoptosis associated with the effect of tetrandrine were also investigated in HaCaT cells.

Materials and Methods

Chemicals and reagents. Tetrandrine, dimethyl sulfoxide, propidium iodide (PI), and trypsin-ethylenediamine tetra-acetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, L-glutamine, and antibiotic (penicillin/streptomycin) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies to superoxide dismutase (SOD) (Cu/Zn), SOD (Mn), catalase, and β -actin were obtained from Santa Cruz Biotechnology; antibodies to caspase-3, BCL2 apoptosis regulator-associated X-protein (BAX), and BCL2 were from Cell Signaling Technology, Inc. (Beverly, MA, USA); and those against poly ADP ribose polymerase (PARP), ataxia telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3 related (ATR), P53, and phospho-P53 (p-P53) were from Calbiochem (San Diego, CA, USA); anti-phosphorylated histone H2AX (p-H2A.X) was from GeneTex Inc. (Irvine, CA, USA). The secondary antibody (anti-IgG) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Tetrandrine stock solutions were dissolved in dimethyl sulfoxide and further diluted in the culture medium.

Cell culture. HaCaT Human keratinocyte cells were kindly provided by Professor Huey-Chun Huang (China Medical University, Taiwan, ROC). HaCaT cells were cultured in DMEM supplemented with 1% antibiotic (100 units/ml penicillin and 100 μ g/ml streptomycin), 10% fetal bovine serum, and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO₂.

Examination of cell morphological changes and cell viability. HaCaT cells were cultured to 80% confluence and harvested (passage 2). Cells (1×10^5 cells/ml) were cultured in 12-well plates with DMEM overnight. Cells were treated with DMSO (as control group), tetrandrine at 20 μ M, H_2O_2 at 500 μ M, or pre-treated with 20 μ M tetrandrine for 1 h and then treated with 500 μ M H_2O_2 for 3, 6, or 12 h. After treatment, cells were observed under phase-contrast microscopy, and cell viability examination was performed by PI exclusion assay as described previously (38).

Annexin V/PI staining. Annexin V/PI double staining assay was used to measure apoptotic cell death as described previously (38). HaCaT cells (1×10^5 cells/ml) were plated in 12-well plates for 24 h. The cells were treated with DMSO, 500 μ M H_2O_2 or pre-treated with 20 μ M tetrandrine for 1 h and then treated with 500 μ M H_2O_2 for 12 h. After treatment, cells from individual wells were collected, resuspended in annexin V binding buffer, and incubated with annexin V/PI in the dark for 15 min. All cells from each treatment were collected and further analyzed using BD FACSCalibur (BD Biosciences, San Jose, CA, USA) for determining apoptotic cell numbers. Experiments were performed in triplicate.

4',6-Diamidino-2-phenylindole (DAPI) assay. HaCaT cells (1×10^5 cells/ml) were treated with DMSO, 500 μ M H_2O_2 , or pre-treated

Figure 1. Tetrandrine affects cell morphology and cell viability in HaCaT cells. HaCaT cells were treated with H₂O₂ (500 μM) or tetrandrine (20 μM), or treated with tetrandrine (20 μM) for 1 h and then with H₂O₂ (500 μM) for 3, 6 or 12 h. Cells were monitored and photographed under phase-contrast microscopy (A), and total cell viability was calculated by propidium iodide exclusion assay (B) as described in the Materials and Methods. Different letters indicate significant differences ($p < 0.05$) between groups by one-way analysis of variance.

with 20 μM tetrandrine for 1 h and then treated with 500 μM H₂O₂ for 12 h. Subsequently, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and stained with DAPI solution (2 μg/ml) and examined and photographed under a fluorescence microscope as described previously (39).

Western blotting analysis. HaCaT cells (3×10^6 cells/dish) were cultured onto 10-cm dishes overnight and treated with DMSO, 500 μM H₂O₂ or pre-treated with 20 μM tetrandrine for 1 h and then treated with 500 μM H₂O₂ for 12 h. At the end of incubation, cells were collected, total proteins were extracted, and their protein

concentration was quantified by Bio-Rad protein assay kit (Hercules, CA, USA) (40-42). A defined amount of sample (30 μg) from each treatment was separated by 10% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore, Belford, MA, USA). Subsequently, the membranes were reacted with primary antibodies against SOD (Cu/Zn), SOD (Mn), catalase, ATM, ATR, p-H2A.X, p-P53, P53, BAX, BCL2, caspase-3, caspase-8, caspase-9, PARP, and β-actin at 4°C overnight. Membranes were then washed with PBS with Tween® 20, incubated with peroxidase-conjugated anti-mouse IgG, and proteins bands were visualized by their chemiluminescence signals using enhanced chemiluminescent

Figure 2. Tetrandrine induced apoptotic cell death of HaCaT cells. HaCaT cells were placed on a 12-well plate overnight and treated with or without tetrandrine for 1 h and then treated with H_2O_2 (500 μM) for 12 h. Cells were harvested for staining with annexin V/propidium iodide (PI) double staining as described in the Materials and Methods. A: Representative profiles of apoptotic cell death. B: Percentage of apoptotic cell death. *** $p < 0.001$ versus the control, ### $p < 0.001$ versus H_2O_2 alone, as analyzed by one-way analysis of variance.

detection kit (Amersham Biosciences ECLTM, Buckinghamshire, UK) as described previously (40, 43).

Confocal laser scanning microscopy for measuring protein expression. After plating on chambered coverslips at 1×10^5 cells/ml, HaCaT cells were treated with DMSO, 500 μM H_2O_2 or pre-treated with 20 μM tetrandrine for 1 h and then with H_2O_2 for 12 h. Cells were then fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.1% Triton-X 100 in PBS for 15 min. All samples were then washed and stained with primary antibodies to SOD1, catalase, and glutathione. Cells were washed with PBS, stained by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (green fluorescence), and their nucleus was stained by PI (red fluorescence) as described previously (41). Individual samples were photographed for expression of associated proteins under a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

Statistical analysis. The results are presented as the means $\pm$ standard deviation. All samples were obtained three times independently. Statistical analyses were performed by one-way analysis of variance followed by Tukey test for determining significant differences

among groups ($p < 0.05$) using Sigma Plot 12 software (Systat Software, Inc., San Jose, CA, USA).

Results

Tetrandrine altered cell morphology and viability of HaCaT cells. HaCaT cells were treated with tetrandrine or H_2O_2 , alone, or sequentially. Cells were monitored and photographed under phase-contrast microscopy. The cell morphology was unmistakably changed after 12-h treatment (Figure 1A). The total cell viability under each treatment was measured by PI exclusion assay. As shown in Figure 1B, pre-treatment of HaCaT cells with tetrandrine and then H_2O_2 led to a reduction of cell viability (Figure 1B) when compared to cells treated with TET or H_2O_2 alone.

Tetrandrine induced apoptotic cell death of HaCaT cells. As shown in Figure 2, when compared to the control group, H_2O_2 (500 μM) treatment induced 7.2-fold annexin V-

Figure 3. Tetrandrine induced chromatin condensation in HaCaT cells. HaCaT cells (1×10^5 cells/ml) were seeded on chamber coverslips and treated with or without tetrandrine (20 μM) for 1 h then treated with H₂O₂ (500 μM) for 12 h. The cells were then examined and photographed under fluorescent microscopy at $\times 100$ as described in the Materials and Methods. A: Representative images of cell chromatin condensation. B: 2-(4-Amidinophenyl)-6-indolecarbamidine fluorescence intensity in treated cells. *** $p < 0.001$ versus the control, ### $p < 0.001$ versus the H₂O₂ alone, as analyzed by one-way analysis of variance.

positive cells (apoptotic cells), whilst pre-treatment with 20 μM of tetrandrine and then with H₂O₂ induced 9.0-fold annexin V-positive cells. These results indicated that H₂O₂ treated with tetrandrine increased apoptotic cell death and reduced the number of viable cells.

Tetrandrine induced morphological changes and chromatin condensation in nuclei (apoptotic death) of HaCaT cells. After HaCaT cells were pre-treated with tetrandrine and then with H₂O₂, cells were stained with DAPI and photographed under fluorescence microscopy. The results shown in Figure 3 indicate the brighter fluorescence of nuclei in HaCaT cells after pre-treatment with tetrandrine compared with that of H₂O₂ treatment alone. This bright fluorescence indicates that DNA was naked or chromatin was condensed. After calculating the difference in fluorescence (fold of control) between treated and untreated cells, the data indicated that cells pre-treated with tetrandrine had a 2.6-fold higher intensity than those under H₂O₂ treatment.

Tetrandrine affects ROS production, DNA damage, and expression of apoptosis-associated proteins in HaCaT cells. As shown in Figure 4, when compared to the control group, pre-treatment with tetrandrine then with H₂O₂ significantly increased the production of ROS-associated proteins such as SOD (Cu/Zn) and SOD (Mn) but significantly reduced the level of catalase (Figure 4A). It also increased the expression of DNA repair-associated proteins ATM, ATR, p-P53, P53, and p-H2A.X when compared to the control group (Figure 4B). Furthermore, such treatment increased the levels of pro-apoptotic proteins BAX, caspase-3, caspase-8, caspase-9, and PARP in HaCaT cells, whilst that of anti-apoptotic protein BCL2 did not decrease when compared to the control group (Figure 4C).

Tetrandrine affects the expression and the nuclear translocation of SOD1, catalase, and glutathione in H₂O₂-treated HaCaT cells. The results from western blotting indicated that pre-treatment of tetrandrine then treatment with H₂O₂ significantly increased SOD (Cu/Zn) and SOD

Figure 4. Tetrandrine enhanced expression of reactive oxygen species, DNA damage, and apoptosis-associated proteins in HaCaT cells. HaCaT cells were treated with or without tetrandrine at 20 μM for 1 h then treated with H₂O₂ (500 μM) for 12 h. Cells from each treatment were harvested for determining expression of apoptotic proteins by western blotting as described in the Materials and Methods. A: Superoxide dismutase (SOD) (Cu/Zn), SOD (Mn) and catalase. B: phospho-ataxia telangiectasia mutated (p-ATM), phospho- ataxia-telangiectasia and Rad3 related (p-ATR), P53, phospho-P53 (p-P53), and phosphorylated histone H2AX (p-H2A.X). C: BCL2 apoptosis regulator (BCL2)-associated X-protein (BAX), BCL2, caspase-9, caspase-8, caspase-3, and poly ADP ribose polymerase (PARP).

(Mn) but reduced catalase (Figure 4A) in HaCaT cells. In order to further confirm these associations of H₂O₂ treatment and their effects on SOD and glutathione expression, confocal laser microscopy was used to measure expression of both proteins in HaCaT cells. After cells were pre-treated with or without tetrandrine and treated with H₂O₂ (500 μM) for 12 h, cells were probed with SOD1, catalase, and glutathione antibodies, and then examined, observed and photographed under confocal laser microscopy. As shown in Figure 5, tetrandrine promoted the expression and nuclear translocation of SOD1 and glutathione but reduced the

expression of catalase in HaCaT cells, indicating these observations are in agreement with the results from the western blotting assay.

Discussion

It is well documented that H₂O₂, an inducer of ROS (42, 44), also induces cytotoxic effects which cause cell death *via* induction of apoptosis, depending on the dose (39, 45). Cells produce ROS such as O₂⁻, OH⁻, and H₂O₂ in physiological intracellular reactions and functions in cellular metabolism,

Figure 5. Tetrandrine affected expression and location of reactive oxygen species-associated proteins in HaCaT cells. HaCaT cells (1×10^5 cells/ml) were maintained on coverslips and pre-treated with tetrandrine at 20 μ M for 1 h then treated with or without H₂O₂ (500 μ M) for 12 h. Cells were fixed with 4% formaldehyde, permeabilized by using 0.2% Triton-X 100 for 15 min, and were probed with primary antibodies to SOD1 (A), catalase (B), and glutathione (C) and by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (green fluorescence), and the nucleus was stained by propidium iodide (red fluorescence) as described in the Materials and Methods.

and these act as mediators of cell signaling pathways (46). Antioxidants are able to attenuate the damaging effects of ROS, and tetrandrine has been suggested to have potential as an antioxidant drug in (47). Studies have shown that tetrandrine induces cytotoxic effects through the induction of apoptosis of many types of human cancer cells *in vitro*

(48, 49). The relationship between tetrandrine- and H₂O₂-induced cell death remains underexplored. Herein, we found that H₂O₂ induced morphological cell changes and reduced total viable HaCaT cells *in vitro*. However, HaCaT cells pre-treated with tetrandrine and then treated with H₂O₂ led to increasing morphological changes and increased cell death

Figure 6. The possible molecular mechanism involved in apoptotic cell death by tetrandrine pre-treatment and then H_2O_2 treatment of HaCaT cells. p-ATM: Phosphor-ataxia telangiectasia mutated; p-ATR: phospho-ataxia-telangiectasia and Rad3-related; BAX: BCL2 apoptosis regulator-associated X-protein; p-H2A.X: phosphorylated histone H2AX; p-P53: phospho-P53; PARP: poly ADP ribose polymerase; ROS: reactive oxygen species; SOD: superoxide dismutase.

(Figure 1). Thus, tetrandrine augmented the H_2O_2 -induced cytotoxic effect. Another reason for selecting H_2O_2 for this study is that H_2O_2 -induced apoptosis of retinal pigmented epithelial cells is a well-known study model for drug discovery (50-53), so our model system is similar.

Alone, tetrandrine and H_2O_2 both induce cancer cell apoptosis (30-32, 45, 46). Therefore, in order to further understand whether tetrandrine enhances H_2O_2 induced cytotoxic effects (reduced total viable cell number) in HaCaT cells through apoptotic cell death, annexin V/PI double staining assay was used to measure cell apoptosis. Annexin V/PI double staining assay indicated that H_2O_2 induced cell apoptosis (Figure 2A) and tetrandrine indeed enhanced H_2O_2 -induced cell apoptosis (Figure 2B) in HaCaT cells. DAPI staining assay confirmed that tetrandrine also significantly enhanced H_2O_2 -induced chromatin condensation (one of the characteristics of cell apoptosis) (Figure 3) in HaCaT cells. Both results are in agreement for tetrandrine enhancing H_2O_2 -induced cell apoptosis in HaCaT cells *in vitro*.

We used western blotting assay for examining cellular protein expression in HaCaT cells in order to elucidate the possible molecular mechanisms involved in apoptosis induction. Pre-treatment of HaCaT cells with tetrandrine significantly enhanced H_2O_2 induced protein expression of pro-apoptotic BAX, caspase-3, caspase-8, caspase-9, and PARP but reduced that of anti-apoptotic protein BCL2 (Figure 4C). The anti-apoptotic BCL2-family proteins and apoptotic BAX protein

play important roles in mitochondrion-dependent extrinsic and intrinsic cell death pathways (54, 55). BAX induces release of cytochrome c from mitochondria, resulting in activation of caspase-9, PARP and caspase-3 for the development of apoptosis (54, 55). Caspase-3, BAX, BCL2, and cytochrome c are well-known markers of apoptosis. Our results also showed that pre-treatment with tetrandrine significantly enhanced expression of H_2O_2 -increased DNA repair-associated proteins, including ATM, ATR, p-P53, P53, and p-H2A.X (Figure 4B). Upon DNA damage, P53 expression and activation increases, resulting in repair of DNA damage or leading to cell death. The DNA repair-associated proteins such as ATM, ATR, and p-H2A.X that in these studies were increased (Figure 4B). ATR is closely related to two other DNA damage response kinases (ATM, and DNA-PK), these kinases responding to different DNA damage insults, that are primarily double-strand breaks for ATM and DNA-PK, and replication stress for ATR (56). γ H2A.X expression was increased in HaCaT cells after pre-treatment with tetrandrine and treatment with H_2O_2 . γ H2A.X is a marker associated with DNA damage and repair (57).

Psoriasis is a skin disorder that shows a marked epidermal hyper-proliferation of keratinocytes, aberrant differentiation, and keratinocyte inflammation (58, 59). Herein, our results indicated that tetrandrine enhanced H_2O_2 -induced apoptotic cell death of keratinocytes, thus, we suggest tetrandrine combined with H_2O_2 may be a potent approach for treating diseases involving hyper-proliferation of keratinocytes.

Numerous studies have shown that many diseases such as cancer, diabetes, and Parkinson's disease are associated with intracellular H_2O_2 generated *via* extracellular H_2O_2 (60-62). Antioxidants extracted from natural products can reduce skin injury, aging, and cancer risk based on their antioxidant activities (63, 64). Tetrandrine was reported to increase the expression of antioxidative enzymes (SOD and glutathione) in animal studies (65). Tetrandrine is one of the major components in traditional Chinese herbal medicine and has been shown to attenuate oxidative stress. Catalase, SOD, and glutathione peroxidase are major enzymes for effectively scavenging ROS and attenuating oxidative damage (66, 67). Catalase can break down H_2O_2 into its inert components, which has been associated with the determining the cancer-killing ability of natural products (68). Furthermore, H_2O_2 has been applied to different cell types, and can reduce cell viability, depending on a decrease in the concentration of catalase (69). Based on the results from our confocal laser microscopy study, tetrandrine pre-treatment and then treatment with H_2O_2 reduced H_2O_2 -induced expression of catalase; however, it increased that of SOD(Cu/Zn) and SOD(Mn) (Figure 4A) in HaCaT cells. These results are in agreement with the results from confocal laser microscopy (Figure 5A).

In the present work, pre-treatment of HaCaT cells with tetrandrine significantly subsequently enhanced H_2O_2 -induced cell apoptosis, through the molecular mechanism

shown in Figure 6. These findings indicate a potential approach for the therapy of diseases associated with keratinocyte proliferation.

Conflicts of Interest

The Authors confirm that there are no conflicts of interest in regard to this study.

Authors' Contributions

Study conception and design: YCC, FSC and WWH. Acquisition of data: YCC, CLK, SYH, TDW, CLC and JCC. Analysis and interpretation of data: YCC, KCL, SFP and WJH. Drafting of article: YCC, FSC and WWH. Critical revision: YCC and WWH. All Authors discussed the results and commented on the article.

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References

- Costa MF, Durço AO, Rabelo TK, Barreto RSS and Guimaraes AG: Effects of carvacrol, thymol and essential oils containing such monoterpenes on wound healing: A systematic review. *J Pharm Pharmacol* 71(2): 141-155, 2019. PMID: 30537169. DOI: 10.1111/jphp.13054
- Di Meglio P, Duarte JH, Ahlfors H, Owens ND, Li Y, Villanova F, Tosi I, Hirota K, Nestle FO, Mrowietz U, Gilchrist MJ and Stockinger B: Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions. *Immunity* 40(6): 989-1001, 2014. PMID: 24909886. DOI: 10.1016/j.immuni.2014.04.019
- Furue M, Tsuji G, Mitoma C, Nakahara T, Chiba T, Morino-Koga S and Uchi H: Gene regulation of filaggrin and other skin barrier proteins *via* aryl hydrocarbon receptor. *J Dermatol Sci* 80(2): 83-88, 2015. PMID: 26276439. DOI: 10.1016/j.jdermsci.2015.07.011
- de Gruijl FR: Action spectrum for photocarcinogenesis. *Recent Results Cancer Res* 139: 21-30, 1995. PMID: 7597292. DOI: 10.1007/978-3-642-78771-3_2
- Young AR, Chadwick CA, Harrison GI, Nikaido O, Ramsden J and Potten CS: The similarity of action spectra for thymine dimers in human epidermis and erythema suggests that DNA is the chromophore for erythema. *J Invest Dermatol* 111(6): 982-988, 1998. PMID: 9856805. DOI: 10.1046/j.1523-1747.1998.00436.x
- Hyun YJ, Piao MJ, Kang KA, Zhen AX, Madushan Fernando PDS, Kang HK, Ahn YS and Hyun JW: Effect of fermented fish oil on fine particulate matter-induced skin aging. *Mar Drugs* 17(1):61, 2019. PMID: 30669248. DOI: 10.3390/md17010061
- Li D, Li Y, Li G, Zhang Y, Li J and Chen H: Fluorescent reconstitution on deposition of PM_{2.5} in lung and extrapulmonary organs. *Proc Natl Acad Sci USA* 116(7): 2488-2493, 2019. PMID: 30692265. DOI: 10.1073/pnas.1818134116
- Pan TL, Wang PW, Aljuffali IA, Huang CT, Lee CW and Fang JY: The impact of urban particulate pollution on skin barrier function and the subsequent drug absorption. *J Dermatol Sci* 78(1): 51-60, 2015. PMID: 25680853. DOI: 10.1016/j.jdermsci.2015.01.011
- Segre JA: Epidermal barrier formation and recovery in skin disorders. *J Clin Invest* 116(5): 1150-1158, 2006. PMID: 16670755. DOI: 10.1172/JCI28521
- Lewis DA, Yi Q, Travers JB and Spandau DF: UVB-induced senescence in human keratinocytes requires a functional insulin-like growth factor-1 receptor and p53. *Mol Biol Cell* 19(4): 1346-1353, 2008. PMID: 18216278. DOI: 10.1091/mbc.e07-10-1041
- Rauhala L, Hämäläinen L, Salonen P, Bart G, Tammi M, Pasonen-Seppänen S and Tammi R: Low dose ultraviolet B irradiation increases hyaluronan synthesis in epidermal keratinocytes *via* sequential induction of hyaluronan synthases Has1-3 mediated by p38 and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) signaling. *J Biol Chem* 288(25): 17999-18012, 2013. PMID: 23645665. DOI: 10.1074/jbc.M113.472530
- Moore C, Cevikbas F, Pasolli HA, Chen Y, Kong W, Kempkes C, Parekh P, Lee SH, Kontchou NA, Yeh I, Jokerst NM, Fuchs E, Steinhoff M and Liedtke WB: UVB radiation generates sunburn pain and affects skin by activating epidermal TRPV4 ion channels and triggering endothelin-1 signaling. *Proc Natl Acad Sci USA* 110(34): E3225-E3234, 2013. PMID: 23929777. DOI: 10.1073/pnas.1312933110
- Kim KE, Cho D and Park HJ: Air pollution and skin diseases: Adverse effects of airborne particulate matter on various skin diseases. *Life Sci* 152: 126-134, 2016. PMID: 27018067. DOI: 10.1016/j.lfs.2016.03.039
- Nakashima Y, Ohta S and Wolf AM: Blue light-induced oxidative stress in live skin. *Free Radic Biol Med* 108: 300-310, 2017. PMID: 28315451. DOI: 10.1016/j.freeradbiomed.2017.03.010
- Albanesi C, Scarponi C, Giustizieri ML and Girolomoni G: Keratinocytes in inflammatory skin diseases. *Curr Drug Targets Inflamm Allergy* 4(3): 329-334, 2005. PMID: 16101542. DOI: 10.2174/1568010054022033
- Gonzalez C, Sanz-Alfayate G, Agapito MT, Gomez-Niño A, Rocher A and Obeso A: Significance of ROS in oxygen sensing in cell systems with sensitivity to physiological hypoxia. *Respir Physiol Neurobiol* 132(1): 17-41, 2002. PMID: 12126693. DOI: 10.1016/s1569-9048(02)00047-2
- Bottai G, Mancina R, Muratori M, Di Gennaro P and Lotti T: 17β-estradiol protects human skin fibroblasts and keratinocytes against oxidative damage. *J Eur Acad Dermatol Venereol* 27(10): 1236-1243, 2013. PMID: 22988828. DOI: 10.1111/j.1468-3083.2012.04697.x
- Silva SAME, Michniak-Kohn B and Leonardi GR: An overview about oxidation in clinical practice of skin aging. *An Bras Dermatol* 92(3): 367-374, 2017. PMID: 29186250. DOI: 10.1590/abd1806-4841.20175481
- Park WH: MAPK inhibitors, particularly the JNK inhibitor, increase cell death effects in H₂O₂-treated lung cancer cells *via* increased superoxide anion and glutathione depletion. *Oncol Rep* 39(2): 860-870, 2018. PMID: 29207156. DOI: 10.3892/or.2017.6107
- Yan S, Sorrell M and Berman Z: Functional interplay between ATM/ATR-mediated DNA damage response and DNA repair pathways in oxidative stress. *Cell Mol Life Sci* 71(20): 3951-3967, 2014. PMID: 24947324. DOI: 10.1007/s00018-014-1666-4

- 21 Friedberg EC: DNA damage and repair. *Nature* 421(6921): 436-440, 2003. PMID: 12540918. DOI: 10.1038/nature01408
- 22 Zuliani T, Denis V, Noblesse E, Schnebert S, Andre P, Dumas M and Ratinaud MH: Hydrogen peroxide-induced cell death in normal human keratinocytes is differentiation dependent. *Free Radic Biol Med* 38(3): 307-316, 2005. PMID: 15629860. DOI: 10.1016/j.freeradbiomed.2004.09.021
- 23 Luo Z, Xu X, Sho T, Zhang J, Xu W, Yao J and Xu J: ROS-induced autophagy regulates porcine trophectoderm cell apoptosis, proliferation, and differentiation. *Am J Physiol Cell Physiol* 316(2): C198-C209, 2019. PMID: 30485137. DOI: 10.1152/ajpcell.00256.2018
- 24 Bhagya N and Chandrashekar KR: Tetrandrine – A molecule of wide bioactivity. *Phytochemistry* 125: 5-13, 2016. PMID: 26899361. DOI: 10.1016/j.phytochem.2016.02.005
- 25 Meng LH, Zhang H, Hayward L, Takemura H, Shao RG and Pommier Y: Tetrandrine induces early G1 arrest in human colon carcinoma cells by down-regulating the activity and inducing the degradation of G1-S-specific cyclin-dependent kinases and by inducing p53 and p21Cip1. *Cancer Res* 64(24): 9086-9092, 2004. PMID: 15604277. DOI: 10.1158/0008-5472.CAN-04-0313
- 26 Gao LN, Feng QS, Zhang XF, Wang QS and Cui YL: Tetrandrine suppresses articular inflammatory response by inhibiting pro-inflammatory factors via NF- κ B inactivation. *J Orthop Res* 34(9): 1557-1568, 2016. PMID: 26748661. DOI: 10.1002/jor.23155
- 27 Lai JH: Immunomodulatory effects and mechanisms of plant alkaloid tetrandrine in autoimmune diseases. *Acta Pharmacol Sin* 23(12): 1093-1101, 2002. PMID: 12466046.
- 28 Zhang HN, Xin HT, Zhang WD, Jin CJ, Huang SY and Zhang Y: [The anti-fibrotic effects of Qidan granule in experimental silicosis]. *Zhonghua Yu Fang Yi Xue Za Zhi* 41(4): 290-294, 2007. PMID: 17959051.
- 29 Liu C, Gong K, Mao X and Li W: Tetrandrine induces apoptosis by activating reactive oxygen species and repressing Akt activity in human hepatocellular carcinoma. *Int J Cancer* 129(6): 1519-1531, 2011. PMID: 21128229. DOI: 10.1002/ijc.25817
- 30 Bhagya N, Chandrashekar KR, Prabhu A and Rekha PD: Tetrandrine isolated from *Cyclea peltata* induces cytotoxicity and apoptosis through ROS and caspase pathways in breast and pancreatic cancer cells. *In Vitro Cell Dev Biol Anim* 55(5): 331-340, 2019. PMID: 30945115. DOI: 10.1007/s11626-019-00332-9
- 31 Bai XY, Liu YG, Song W, Li YY, Hou DS, Luo HM and Liu P: Anticancer activity of tetrandrine by inducing pro-death apoptosis and autophagy in human gastric cancer cells. *J Pharm Pharmacol* 70(8): 1048-1058, 2018. PMID: 29770446. DOI: 10.1111/jphp.12935
- 32 Chen Z, Zhao L, Zhao F, Yang G and Wang JJ: Tetrandrine suppresses lung cancer growth and induces apoptosis, potentially via the VEGF/HIF-1 α /ICAM-1 signaling pathway. *Oncol Lett* 15(5): 7433-7437, 2018. PMID: 29849794. DOI: 10.3892/ol.2018.8190
- 33 Lien JC, Lin MW, Chang SJ, Lai KC, Huang AC, Yu FS and Chung JG: Tetrandrine induces programmed cell death in human oral cancer CAL 27 cells through the reactive oxygen species production and caspase-dependent pathways and associated with beclin-1-induced cell autophagy. *Environ Toxicol* 32(1): 329-343, 2017. PMID: 26822499. DOI: 10.1002/tox.22238
- 34 Ng LT, Chiang LC, Lin YT and Lin CC: Antiproliferative and apoptotic effects of tetrandrine on different human hepatoma cell lines. *Am J Chin Med* 34(1): 125-135, 2006. PMID: 16437745. DOI: 10.1142/S0192415X06003692
- 35 Liu KC, Lin YJ, Hsiao YT, Lin ML, Yang JL, Huang YP, Chu YL and Chung JG: Tetrandrine induces apoptosis in human nasopharyngeal carcinoma NPC-TW 039 cells by endoplasmic reticulum stress and Ca²⁺/Calpain pathways. *Anticancer Res* 37(11): 6107-6118, 2017. PMID: 29061791. DOI: 10.21873/anticancer.12059
- 36 Chen XS, Bao MH and Mei XD: Reversing multidrug resistance of epidermoid carcinoma drug-resistant cell line KB-MRP1 by tetrandrine. *Ai Zheng* 26(8): 846-850, 2007. PMID: 17697545.
- 37 Cui X, Xiao D and Wang X: Inhibition of laryngeal cancer stem cells by tetrandrine. *Anticancer Drugs* 30(9): 886-891, 2019. PMID: 31517730. DOI: 10.1097/CAD.0000000000000803
- 38 Lai KC, Hsu SC, Yang JS, Yu CC, Lein JC and Chung JG: Diallyl trisulfide inhibits migration, invasion and angiogenesis of human colon cancer HT-29 cells and umbilical vein endothelial cells, and suppresses murine xenograft tumour growth. *J Cell Mol Med* 19(2): 474-484, 2015. PMID: 25403643. DOI: 10.1111/jcmm.12486
- 39 Park IJ, Hwang JT, Kim YM, Ha J and Park OJ: Differential modulation of AMPK signaling pathways by low or high levels of exogenous reactive oxygen species in colon cancer cells. *Ann N Y Acad Sci* 1091: 102-109, 2006. PMID: 17341607. DOI: 10.1196/annals.1378.059
- 40 Liu KC, Shih TY, Kuo CL, Ma YS, Yang JL, Wu PP, Huang YP, Lai KC and Chung JG: Sulforaphane induces cell death through G2/M phase arrest and triggers apoptosis in HCT 116 human colon cancer cells. *Am J Chin Med* 44(6): 1289-1310, 2016. PMID: 27627923. DOI: 10.1142/S0192415X16500725
- 41 Cheng ZY, Hsiao YT, Huang YP, Peng SF, Huang WW, Liu KC, Hsia TC, Way TD and Chung JG: Casticin induces DNA damage and affects DNA repair associated protein expression in human lung cancer A549 cells (Running title: casticin induces DNA damage in lung cancer cells). *Molecules* 25(2):341, 2020. PMID: 31952105. DOI: 10.3390/molecules25020341
- 42 Beatty S, Koh H, Phil M, Henson D and Boulton M: The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 45(2): 115-134, 2000. PMID: 11033038. DOI: 10.1016/s0039-6257(00)00140-5
- 43 Lu CC, Yang JS, Chiang JH, Hour MJ, Lin KL, Lee TH and Chung JG: Cell death caused by quinazolinone HMJ-38 challenge in oral carcinoma CAL 27 cells: Dissections of endoplasmic reticulum stress, mitochondrial dysfunction and tumor xenografts. *Biochim Biophys Acta* 1840(7): 2310-2320, 2014. PMID: 24594224. DOI: 10.1016/j.bbagen.2014.02.022
- 44 Pacifici RE and Davies KJ: Protein, lipid and DNA repair systems in oxidative stress: The free-radical theory of aging revisited. *Gerontology* 37(1-3): 166-180, 1991. PMID: 2055497. DOI: 10.1159/000213257
- 45 Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S and Smulson M: Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *J Biol Chem* 274(33): 22932-22940, 1999. PMID: 10438458. DOI: 10.1074/jbc.274.33.22932
- 46 Fridovich I: The biology of oxygen radicals. *Science* 201(4359): 875-880, 1978. PMID: 210504. DOI: 10.1126/science.210504
- 47 Tan LT, Mahendra CK, Yow YY, Chan KG, Khan TM, Lee LH and Goh BH: *Streptomyces* sp. MUM273b: A mangrove-derived potential source for antioxidant and UVB radiation protectants. *Microbiologyopen* 8(10): e859, 2019. PMID: 31199601. DOI: 10.1002/mbo3.859

- 48 Wang CH, Yang JM, Guo YB, Shen J and Pei XH: Anticancer activity of tetrandrine by inducing apoptosis in human breast cancer cell line MDA-MB-231 *in vivo*. *Evid Based Complement Alternat Med* 2020; 6823520, 2020. PMID: 32714412. DOI: 10.1155/2020/6823520
- 49 Jiang L and Hou R: Tetrandrine reverses paclitaxel resistance in human ovarian cancer *via* inducing apoptosis, cell cycle arrest through β -Catenin pathway. *Onco Targets Ther* 13: 3631-3639, 2020. PMID: 32431514. DOI: 10.2147/OTT.S235533
- 50 King RE, Kent KD and Bomser JA: Resveratrol reduces oxidation and proliferation of human retinal pigment epithelial cells *via* extracellular signal-regulated kinase inhibition. *Chem Biol Interact* 151(2): 143-149, 2005. PMID: 15698585. DOI: 10.1016/j.cbi.2004.11.003
- 51 Pittalà V, Fidilio A, Lazzara F, Platania CBM, Salerno L, Foresti R, Drago F and Bucolo C: Effects of novel nitric oxide-releasing molecules against oxidative stress on retinal pigmented epithelial cells. *Oxid Med Cell Longev* 2017: 1420892, 2017. PMID: 29158871. DOI: 10.1155/2017/1420892
- 52 Xie X, Feng J, Kang Z, Zhang S, Zhang L, Zhang Y, Li X and Tang Y: Taxifolin protects RPE cells against oxidative stress-induced apoptosis. *Mol Vis* 23: 520-528, 2017. PMID: 28761325.
- 53 Zhu C, Dong Y, Liu H, Ren H and Cui Z: Hesperetin protects against H₂O₂-triggered oxidative damage *via* upregulation of the Keap1-Nrf2/HO-1 signal pathway in ARPE-19 cells. *Biomed Pharmacother* 88: 124-133, 2017. PMID: 28103505. DOI: 10.1016/j.biopha.2016.11.089
- 54 Brunelle JK and Letai A: Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci* 122(Pt 4): 437-441, 2009. PMID: 19193868. DOI: 10.1242/jcs.031682
- 55 Jourdain A and Martinou JC: Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. *Int J Biochem Cell Biol* 41(10): 1884-1889, 2009. PMID: 19439192. DOI: 10.1016/j.biocel.2009.05.001
- 56 O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA and Goodship JA: A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat Genet* 33(4): 497-501, 2003. PMID: 12640452. DOI: 10.1038/ng1129
- 57 Crowe SL, Movsesyan VA, Jorgensen TJ and Kondratyev A: Rapid phosphorylation of histone H2A.X following ionotropic glutamate receptor activation. *Eur J Neurosci* 23(9): 2351-2361, 2006. PMID: 16706843. DOI: 10.1111/j.1460-9568.2006.04768.x
- 58 Afonina IS, Van Nuffel E and Beyaert R: Immune responses and therapeutic options in psoriasis. *Cell Mol Life Sci* 78(6): 2709-2727, 2021. PMID: 33386888. DOI: 10.1007/s00018-020-03726-1
- 59 Albanesi C, Madonna S, Gisondi P and Girolomoni G: The interplay between keratinocytes and immune cells in the pathogenesis of psoriasis. *Front Immunol* 9: 1549, 2018. PMID: 30034395. DOI: 10.3389/fimmu.2018.01549
- 60 Whittemore ER, Loo DT, Watt JA and Cotman CW: A detailed analysis of hydrogen peroxide-induced cell death in primary neuronal culture. *Neuroscience* 67(4): 921-932, 1995. PMID: 7675214. DOI: 10.1016/0306-4522(95)00108-u
- 61 Evans JL, Maddux BA and Goldfine ID: The molecular basis for oxidative stress-induced insulin resistance. *Antioxid Redox Signal* 7(7-8): 1040-1052, 2005. PMID: 15998259. DOI: 10.1089/ars.2005.7.1040
- 62 Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* 8(7): 579-591, 2009. PMID: 19478820. DOI: 10.1038/nrd2803
- 63 Sawicka D, Car H, Borawska MH and Nikliński J: The anticancer activity of propolis. *Folia Histochem Cytobiol* 50(1): 25-37, 2012. PMID: 22532133. DOI: 10.2478/18693
- 64 Zhou Y, Zheng J, Li Y, Xu DP, Li S, Chen YM and Li HB: Natural polyphenols for prevention and treatment of cancer. *Nutrients* 8(8): 515, 2016. PMID: 27556486. DOI: 10.3390/nu8080515
- 65 Wang X, Yang Y, Yang D, Tong G, Lv S, Lin X, Chen C and Dong W: Tetrandrine prevents monocrotaline-induced pulmonary arterial hypertension in rats through regulation of the protein expression of inducible nitric oxide synthase and cyclic guanosine monophosphate-dependent protein kinase type 1. *J Vasc Surg* 64(5): 1468-1477, 2016. PMID: 26527422. DOI: 10.1016/j.jvs.2015.09.016
- 66 Di Mambro VM, Borin MF and Fonseca MJ: Topical formulations with superoxide dismutase: influence of formulation composition on physical stability and enzymatic activity. *J Pharm Biomed Anal* 32(1): 97-105, 2003. PMID: 12852452. DOI: 10.1016/s0731-7085(03)00055-4
- 67 Shamsi FA, Chaudhry IA, Boulton ME and Al-Rajhi AA: L-carnitine protects human retinal pigment epithelial cells from oxidative damage. *Curr Eye Res* 32(6): 575-584, 2007. PMID: 17612973. DOI: 10.1080/02713680701363833
- 68 Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, Tolliday NJ, Golub TR, Carr SA, Shamji AF, Stern AM, Mandinova A, Schreiber SL and Lee SW: Selective killing of cancer cells by a small molecule targeting the stress response to ROS. *Nature* 475(7355): 231-234, 2011. PMID: 21753854. DOI: 10.1038/nature10167
- 69 Klingelhöffer C, Kämmerer U, Koospal M, Mühling B, Schneider M, Kapp M, Kübler A, Germer CT and Otto C: Natural resistance to ascorbic acid induced oxidative stress is mainly mediated by catalase activity in human cancer cells and catalase-silencing sensitizes to oxidative stress. *BMC Complement Altern Med* 12: 61, 2012. PMID: 22551313. DOI: 10.1186/1472-6882-12-61

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