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Stellettin A Induces Endoplasmic Reticulum Stress in Murine B16 Melanoma Cells

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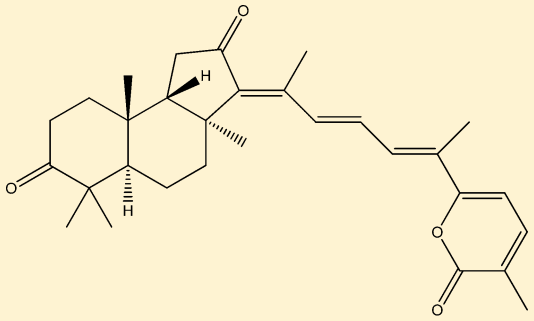
ABSTRACT: Isomalabaricanes are a small class of rearranged triterpenoids obtained from marine sponges. Most of these are cytotoxic to tumor cells, but the underlying mechanism is not clear. In this study, it was demonstrated that stellettin A (**1**), obtained from *Geodia japonica*, inhibited the growth of B16F10 murine melanoma cells by the induction of endoplasmic reticulum stress and accumulation of unfolded proteins. Immunoblotting analysis revealed abnormal glycosylation patterns of two melanoma marker proteins, tyrosinase and tyrosinase-related protein 1, and the retention of these proteins in the endoplasmic reticulum. Compound **1** induced the upregulation of the unfolded protein chaperone, glucose-regulated protein 78, in a dose-dependent manner. Increase of autophagosome-associated protein light chain 3 (LC3) in a membrane-bound form (LC3II) and its immunofluorescence co-localization with tyrosinase suggest the possible removal of deglycosylated and unfolded proteins by autophagy of the cells. There was no change in either the expression of the apoptosis marker protein Bcl-2 or the appearance of apoptotic nuclei in **1**-treated cells. Taken together, **1** is an endoplasmic reticulum stressor that inhibits the growth of B16 melanoma cells by induction of abnormal protein glycosylation and autophagy.



stelletin A (1)

Melanoma is a common type of skin disease that causes about 65% of the mortality by skin cancers.[1] The incidence of melanoma is increasing in Caucasian populations by 2−5% annually.[2] Surgical procedures followed by adjuvant immunotherapy is a standard therapy, but median survival times were reported to be about eight months for Caucasians[3] and 12 months for Asians in Taiwan.[4] The five-year survival of patients with malignant melanoma ranges from 5% to 10% due to chemoresistance.[3,5] New strategies targeting specific molecular events to block melanoma development have been suggested, and current chemotherapeutic agents, such as fenretinide, induce tumor cell death through the activation of endoplasmic reticulum stress.[6] There remains a pressing need for further effective medication for metastatic melanoma.[7]

Isomalabaricanes belong to a small group of rearranged triterpene metabolites biosynthesized by marine sponges such as those in the genera *Stelletta*,[8] *Jaspis*,[9] *Geodia*,[10] and *Rhabdastrella*.[11−13] In tumor cells, these compounds have been found to generate reactive oxygen species (ROS),[14−16] arrest proliferating cells,[17] and induce apoptosis.[14−16] Among these cytotoxic compounds, the stellettins displayed cytotoxicity in the 60-cell assay panel of the National Cancer Institute,[18] whereas stellettin A (**1**) was found to induce apoptosis through a ROS-mitochondria-caspase pathway.[15] Rhabdastrellic acid A, another member of the isomalabaricane family isolated from *Rhabdastrella globostellata*, was demonstrated to trigger autophagy and induce caspase-independent cell death in Hep3B and A549 cells.[19]

In a continuing effort to study the underlying mechanisms of natural cytotoxic compounds, the abnormal glycosylation of melanoma marker proteins and the induction of endoplasmic reticulum stress, culminating in autophagic changes, have been observed in murine B16 melanoma cells following treatment with **1**, as described herein.

RESULTS AND DISCUSSION

In a comparative 48 h cytotoxicity study of stellettin A (**1**) against five cell lines, the highest sensitivity was observed in murine B16 melanoma cells, showing an IC_{50} value of 0.15 μg/mL (Figure 1a), followed by TM3 cells (IC_{50} 0.8 μg/mL). The other three cell lines (human immortalized HaCaT keratinocytes, human colon HT29 carcinoma cells, and mouse melanocyte melan-a cells) were much less sensitive, by at least an order of magnitude (Figure 1a). The IC_{50} value of **1** for B16 cells at 24 h was about 2 μg/mL (Figure 1b). Due to the susceptibility of B16 cells, they were used in subsequent studies.

B16 cells are melanoma cells derived from C57BL/6J mice. Many melanoma research and pigmentation studies have been conducted using this cell line, taking advantage of their

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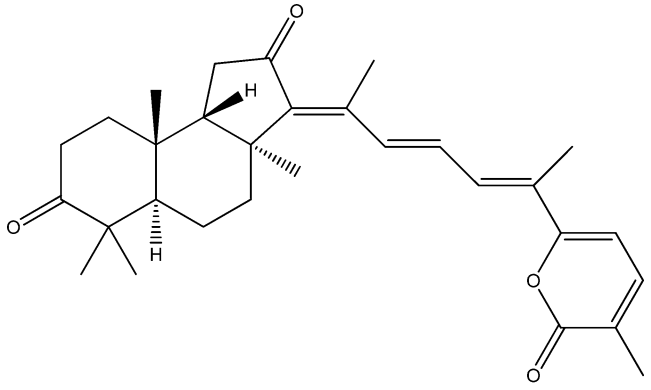

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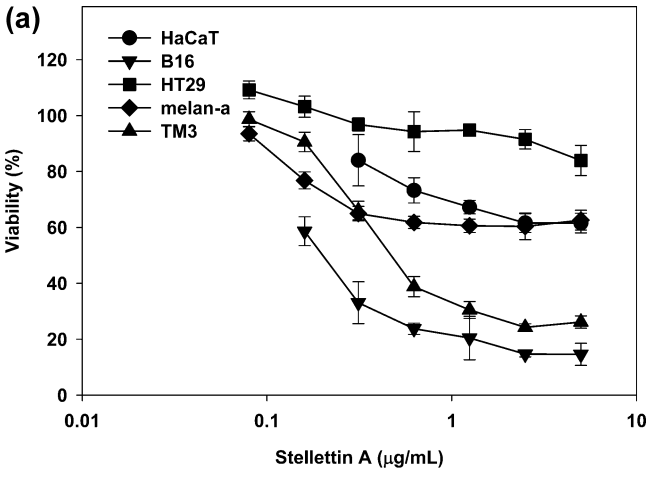

Figure 1. Cytotoxicity assays of stellettin A (**1**) in five cell lines. (a) Cells (10 000/0.1 mL for B16 and TM3 cells and 20 000/0.1 mL for HaCaT, HT29, and melan-a cells) seeded in each well of 96-well plates and treated with different concentrations of **1** for 48 h, before being subjected to sulforhodamine B (SRB) colorimetric measurement. (b) B16 cells (20 000/0.1 mL) treated with serial concentrations of **1** for 24 or 48 h, before being subjected to a SRB colorimetric assay. Results are presented as means and standard deviations of triplicates.

constitutive expression of the marker proteins for melanogenesis, i.e., tyrosinase (TYR) and tyrosinase-related protein 1 (TRP-1). Both TYR and TRP-1 are glycoproteins biosynthesized in the ribosome, matured in the endoplasmic reticulum (ER), and transported to the Golgi en route to their target organelle, the melanosome.[20] Nascent TYR and TRP-1 polypeptides in the monoglucosylated form are coupled with the ER chaperone, calnexin, for proper protein folding. Defective proteins, e.g., misfolded or unfolded TYR and TRP-1, will remain in the ER or the Golgi leading to ER- or Golgi-retention and stress, a process that triggers autophagy, senescence, or apoptosis.[21]

TRP-1 is a cofactor that forms a complex with TYR during the process of melanogenesis. Depletion of TRP-1 by antisense TRP-1 transfection in melanoma cells decreases pigment formation and leads to cell-cycle arrest at the G1 phase, followed by apoptosis.[22] Indeed, in the present study, depigmentation was observed in B16 cells after treatment with **1** for 48 h (unpublished data). In the present investigation, immunoblotting analysis in untreated B16 cells revealed two major bands at about 80 kDa due to the mature form of TRP-1. Following treatment with **1** at 0.6 μg/mL for 24 h, the cells exhibited two additional bands of smaller size (about 70 kDa), representing the immature and deglycosylated form of TRP-1 (Figure 2a). It is known that *N*-glycan processing and maturation of TYR and TRP-1 occur in the ER, and these mature glycoproteins are not sensitive to endoglycosidase H (EndoH), whereas the immature glycoproteins are cleavable by EndoH, giving rise to bands of smaller molecular size in immunoblotting examination. The immature TYR and TRP-1 remain in the ER and induce an unfold protein response (UPR).[20] While the properly processed glycoproteins in the ER are resistant to EndoH, all types of *N*-glycans formed either in the ER or the Golgi apparatus can be cleaved by the peptide *N*-glycosidase F (PNGase F).[20,23] In order to establish the nature of the TRP-1 species in B16 cells, glycosidase digestions were performed with EndoH and PNGase F. The results showed that the TRP-1 in untreated cells was present mainly in a mature and EndoH-insensitive form of about 80 kDa (Figure 2b), while immature and EndoH-sensitive glycoforms were observed after treatment with **1** for 12−24 h (Figure 2b). The *N*-glycan composition of TRP-1, regardless of being EndoH-sensitive or -insensitive, was confirmed by complete digestion with PNGase F (Figure 2b). A time-course analysis further demonstrated a normal TYR glycosylation pattern in the cells until they had been exposed to **1** for 12 h (Figure 2C).

Both protein deglycosylation and ER retention concomitantly trigger UPR to preserve normal cell functions and survival.[21,24] The UPR-responsive chaperones, glucose-regulatory protein-78 (GRP78) and heat shock cognate protein (HSC70), are expressed in order to prevent the exit of these unfolded proteins from the ER and thus protect cells from apoptosis.[21] The present findings revealed an increasing tendency of both GRP78 and HSC70 proteins after exposure to **1** (0.625 μg/mL) for 12 h. However, a decrease at 24 h (Figure 2c) was observed when the cell viability dropped to 70% (Figure 1b). On the other hand, immunofluorescence of GRP78 was low in untreated cells (Figure 3a), but it increased remarkably in **1**-treated cells in a dose-dependent manner (Figure 3b−e). When the cells are overwhelmed by unfolded proteins or damaged organelles, they undergo autophagy with conversion of a ubiquitin-like molecule, LC3I (microtubule-associated protein 1 light chain 3), to LC3II (Figure 2d)[22] as well as a co-localization of LC3 with TYR as detected in this study (Figure 4). It has been shown that prolonged ER stress would activate the mitochondrial caspase pathway, culminating in either autophagy or apoptosis. The chemotherapeutic agent fenretinide kills melanoma cells by activation of ER stress and apoptotic cell death.[6,25]

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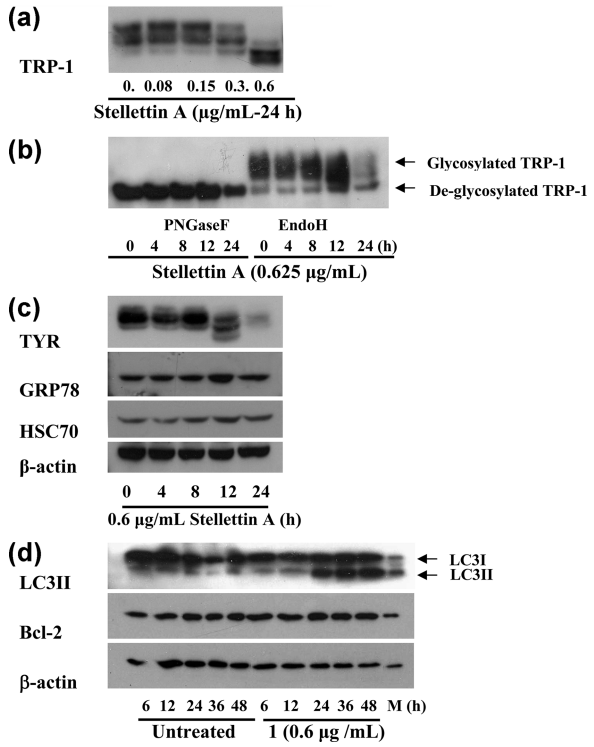

Figure 2. Deglycosylation of TRP-1 in B16 cells treated with stellettin A (**1**). (a) Protein lysates of B16 cells treated with **1** (0−0.625 μg/mL for 24 h) were subjected to Western blotting for the examination of TRP-1. Two major bands of about 80 kDa are present in untreated cells or cells treated with low doses of **1** (<0.3 μg/mL); two additional bands of smaller size (about 70 kDa) representing immature and deglycosylated TRP-1 are present at doses ≥ 0.3 μg/mL. (b) Glycosidase digestion of TRP-1 with EndoH and PGNase F shows that mature and glycosylated TRP-1 is present in untreated B16 cells. Deglycosylated and immature TRP-1 starts to increase after 12 h of treatment. (c) Immunoblotting reveals abnormal glycosylation of TYR after treating with **1** for 12 h. The expression of unfolded GRP78 and HSC70 also increases steadily up to 12 h and declines thereafter, when cell viability drops to about 70%. An equal amount of protein was normalized with β-actin. (d) Time-dependent increase of LC3II and unchanged expression of the apoptotic marker protein Bcl-2 were immunoblotted in B16 cells treated with a low dose (0.6 μg/mL) of **1**. Treatment with 5 mM metformin for 24 h was used as a positive control for the induction of LC3II.[32]

Autophagy is a defensive process of the cell in response to both extrinsic and intrinsic stress whereby unfolded proteins and defective organelles are enclosed by double-membrane vesicles for degradation.[26] Autophagy is induced in response to nutritional starvation, and increasing evidence associates autophagy with an endoplasmic reticulum stress.[27] LC3II is the only well-characterized protein that is localized specifically to the autophagic structures and is thus a biomarker of autophagic change in other studies[28] and also in **1**-treated B16 cells (Figure 2d). It has also been demonstrated that both autophagy and apoptosis occur in cells treated with isomalabaracanes such as rhabdastrellic acid A[19] and **1**,[15] and further study is needed to delineate the role of autophagic change in the cytotoxicity of stellettin A.

Melanoma is characterized by high metastasis, low response rates, and fast development of resistance to chemotherapy. The currently used chemotherapeutic agents, fenretinide and bortezomib, are apoptotic inducers that act through an ER stress-mediated eukaryotic initiation factor 2a-ATF4-caspase pathway.[29] Activation of ER stress has been suggested as a major approach for antimelanoma therapy, which leads the melanoma cells along a degradative pathway.[30] The effective concentrations of these agents range from nanomolar (bortezomib, 200 nM) to micromolar levels (fenretinide, 10 μM).[6] The present findings indicated IC_{50} values of **1** in the range 1.3−4.5 μM (in 24 and 48 h experiments) and, at a micromolar level, that **1** acts as an ER stressor to induce abnormal glycosylation of TYR and TRP-1. Taken together with previous findings of ROS production and apoptotic cell death, stellettin A (**1**) is considered a potential candidate as an antimelanoma agent.

EXPERIMENTAL SECTION

Test Compounds. Stellettin A (**1**) (>98% purity by HPLC), obtained from a South China Sea sponge, *Geodia japonica*,[10] was dissolved in DMSO to make a stock solution of 40 mg/mL, which was then diluted to appropriate concentrations with culture medium before each experiment. The final concentration of DMSO did not exceed 0.5% in any experiment.

Cell Cultures. Except for the immortalized human HaCaT keratinocytes (a gift from Prof. P. Boukamp of the German Cancer Research Center, Heidelberg, Germany) and murine melan-a melanocytes (a gift from Prof. D. C. Bennett of the St. George's Hospital Medical School, London, U.K.), all cell lines, including the human colorectal cancer HT29 cell line (HTB38), murine melanoma B16F10 cell line (CRL6475), and murine Leydig TM3 cells (CRL1714), were obtained from the American Type Culture Collection. Both B16 and HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin sulfate. The TM3 cells were cultured in a supplemented 1:1 mixture of Ham F12 medium and DMEM. The HT29 cells were cultured in supplemented RPMI medium. Melan-a cells were cultured in supplemented RPMI medium with an additional 200 nM PMA (P8139, Sigma) for cell attachment and proliferation.[31] The melan-a, B16, and HaCaT cells used in these experiments were restricted at less than 30 passages, and all cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay. The sulforhodamine B (SRB) colorimetric cytotoxicity assay was adopted in this study.[31] Briefly, cells (5000 cells/

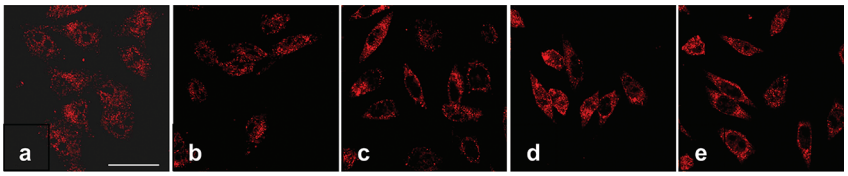

Figure 3. Immunofluorescence of GRP78 in stellettin A (**1**)-treated B16 cells for 24 h. Cells (20 000/mL) were seeded on gelatinized sterilized glass coverslips and treated with serial concentrations of **1** (panels a−e represent concentrations of 0, 0.31, 0.625, 1.25, and 2.5 μg/mL, respectively) for 24 h before fixed with 4% paraformaldehyde and immunostained with an antibody against GRP78. Immunofluorescence was visualized by secondary antibody IgG conjugated to Alexa-594 (red) to show an increase of GRP78 in a dose-dependent manner. (Scale bar = 20 μm.)

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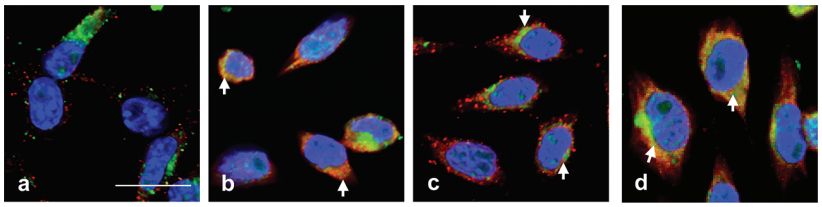

Figure 4. Co-localization (yellow, indicated by arrows) of TYR (green) and LC3 (red) in B16 cells treated with stellettin A (**1**) (panels a–d represent concentrations of 0, 0.625, 1.25, and 2.5 μg/mL, respectively) for 24 h, suggesting the possible removal of deglycosylated TYR by autophagy. Nuclei are stained with DAPI (blue). (Scale bar = 20 μm.)

0.1 mL/well) were treated with a serial dilution of **1** (0.08, 0.156, 0.315, 0.625, 1.25, 2.5, and 5 μg/mL) in 96-well plates (Costar) for 24 or 48 h. The reaction was stopped, and the proteins were fixed by 50% trichloroacetic acid before the cells were stained with 0.4% SRB in 1% acetic acid. The unbound dye was removed, the cells were rinsed with 1% acetic acid five times and air-dried, and the bound dye was dissolved in 100 μL of Tris base (10 mM, pH 10.5). The absorbance was measured with a microtiter plate reader (Molecular Devices, Model Emax) at a wavelength of 570 nm. Data represent mean values and standard deviations of triplicate assays in at least three separate experiments. Since the B16 cells were most sensitive to **1**, subsequent experiments were performed using this cell line.

Fluorescence Staining for Morphological Observation. The B16 cells were seeded on sterile coverslips, treated with a serial dilution of **1** for 24 h, and washed briefly with PBS before they were fixed with paraformaldehyde and stained with antibodies against tyrosinase (13-6800 Zymed Laboratories, South San Francisco, CA, USA) or glucose-regulated protein 78 (GRP78, sc-13968, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by secondary antibody IgG conjugated to AlexaFluor-488 or AlexaFluor-594 in PBS buffer. Chromatin was counterstained with DAPI before the slides were mounted with antifade for confocal microscopy (Axioskop, Zeiss, Japan), using a 450–490 nm excitation block filter and a 520 nm barrier filter.

Immunoblotting Analysis. The B16 cells were exposed to treatment with **1** for 24 h and washed with PBS twice. The total protein lysates were obtained in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.2% Triton X-100, 10 μg/mL aprotinin, and 0.5 mM PMSF) and centrifuged at 10 000 rpm at 4 °C for 10 min. The lysates were normalized for protein content using the BCA protein assay reagent (23223 Pierce, Biotechnology Inc., Rockford, IL, USA). Equal amounts of denatured proteins were loaded and separated on a 10% SDS polyacrylamide gel and then transferred onto a polyvinylene difluoride membrane. After blocking with 2% gelatin, the membrane was stained with specific primary antibodies against (1) the melanogenic proteins tyrosinase (13-6800 Zymed Laboratories, South San Francisco, CA, USA), tyrosinase-related protein-1 (TRP-1, a kind gift of Dr. Vincent Hearing, National Institutes of Health, Bethesda, MD, USA), and β-actin (sc-81178, Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively, and (2) UPR sensor proteins GRP78 and HSC70 (San Cruz Biotechnology), followed by secondary antibody IgG conjugated to horseradish peroxidase in TBS-T buffer. Signals were detected using an ECL Plus Western blotting analysis system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), followed by short exposure to Lumi-film chemiluminescence detection film (Roche Diagnostics Corporation, Indianapolis, IN, USA). Band intensities were quantified by the software PD Quest (BioRad Laboratories, Hercules, CA, USA) and normalized by β-actin.

Glycosylation Analysis. Proteins were extracted with RIPA buffer, and 10 μg of protein lysates were subjected to enzyme digestion by either 100 units of endoglycosidase H (P0702S, New England Biolabs, Beverly, MA, USA) or 100 units of peptide *N*-glycosidase F (P0704S, New England Biolabs) at 37 °C for 1 h, according to the manufacturer's instructions. After incubation, the digested protein lysates were separated on 8% gel and subjected to Western blotting using anti-TRP-1 antibody.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Gleave, M.; Qian, J.; Andreou, C.; Pommerville, P.; Chin, J.; Casey, R.; Steinhoff, G.; Fleshner, N.; Bostwick, D.; Thomas, L.; Rittmaster, R. *Prostate* **2006**, *66*, 1674–1685.
(2) Muenchen, H. J.; Poncza, P. J.; Pienta, K. J. *Urology* **2001**, *57*, 366–370.
(3) Petronilli, V.; Penzo, D.; Scorrano, L.; Bernardi, P.; Di Lisa, F. *J. Biol. Chem.* **2001**, *276*, 12030–12034.
(4) Khosravi-Far, R.; Esposti, M. D. *Cancer Biol. Ther.* **2004**, *3*, 1051–1057.
(5) Boatright, K. M.; Salvesen, G. S. *Curr. Opin. Cell Biol.* **2003**, *15*, 725–731.
(6) Yang, C. L. *Materia Medica of Toxic Herbs*; Chinese Medicine Press: Beijing, 1993; pp 1021–1023.
(7) Ma, Q. G.; Liu, W. Z.; Wu, H. M.; Zhou, T. X.; Qin, G. W. *Phytochemistry* **1997**, *44*, 663–666.
(8) Che, C. T.; Ma, Q. G.; Qin, G. W.; Williams, I. D.; Wu, H. M. *Phytochemistry* **1999**, *52*, 117–121.
(9) Lee, S. H.; Tanaka, T.; Nonaka, G. I.; Nishioka, I.; Zhang, B. *Phytochemistry* **1991**, *30*, 1251–1253.
(10) Tu, S. J.; Wang, P.; Wei, A. *Chin. J. Integr. Trad. West. Med.* **1984**, *4*, 46–47.
(11) Liu, W. K.; Ho, J. C. K. *Biochem. Pharmacol.* **2002**, *63*, 951–957.
(12) Singh, R. P.; Agarwal, R. *Mol. Carcinog.* **2006**, *45*, 436–442.
(13) Liu, X.; Ye, W.; Che, C. T.; Zhao, S. *Zhongcaoyao* **2003**, *34*, 399–401.
(14) Niwa, M.; Tatematsu, H.; Liu, G.; Hirata, Y. *Chem. Lett.* **1984**, *148*, 539–542.
(15) Kim, H. K.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Arch. Pharm. Res.* **1999**, *21*, 406–410.
(16) Banerjee, T.; Valacchi, G.; Ziboh, V. A.; van der Vliet, A. *Mol. Cell. Biochem.* **2002**, *238*, 105–110.
(17) Farombi, E. O.; Moller, P.; Dragsted, L. O. *Cell Biol. Toxicol.* **2004**, *20*, 71–82.
(18) Kito, S.; Shimizu, K.; Okamura, H.; Yoshida, K.; Morimoto, H.; Fujita, M.; Morimoto, Y.; Ohba, T.; Haneji, T. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 950–956.
(19) Coqueret, O. *Gene* **2002**, *299*, 35–55.

(20) Kontopidis, G.; Wu, S. Y.; Zheleva, D. I.; Taylor, P.; McInnes, C.; Lane, D. P.; Fischer, P. M.; Walkinshaw, M. D. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 1871−1876.
(21) Nakamura, K.; Arai, D.; Fukuchi, K. *Arch. Biochem. Biophys.* **2004**, *431*, 47−54.
(22) Li, C. Y.; Gao, T. W.; Wang, G.; Han, Z. Y.; Shen, Z.; Li, T. H.; Liu, Y. F. *Br. J. Dermatol.* **2004**, *150*, 1081−1090.
(23) Bharti, A. C.; Aggarwal, B. B. *Biochem. Pharmacol.* **2002**, *64*, 883−888.
(24) Napoli, C.; de Nigris, F.; Palinski, W. *J. Cell. Biochem.* **2002**, *82*, 674−682.
(25) Suzuki, A.; Tsutomi, N.; Yamamoto, T.; Shibutani, K.; Akahane, K. *Mol. Cell. Biol.* **1999**, *19*, 3842−3847.
(26) Suzuki, A.; Tsutomi, Y.; Akahane, K.; Araki, T.; Miura, M. *Oncogene* **1998**, *17*, 931−939.
(27) Xu, Z. H.; Qin, G. W.; Xu, R. S. *J. Asian Nat. Prod. Res.* **2001**, *3*, 335−340.
(28) Yoshida, M.; Feng, W.; Nishio, K.; Takahashi, M.; Heike, Y.; Saijo, N.; Wakasugi, H.; Ikekawa, T. *Int. J. Cancer* **2001**, *94*, 348−352.
(29) Liu, W .K.; Ho, J. C. K.; Cheung, F. W. K.; Liu, B. P. L; Ye, W. C.; Che, C. T. *Eur. J. Pharmacol.* **2004**, *498*, 71−78.
(30) Liu, W. K.; Ho, J. C. K.; Ng, T. B. *Biochem. Pharmacol.* **2001**, *61*, 33−37.
(31) Sze, S. C. W.; Ho, J. C. K.; Liu, W. K. *J. Cell. Biochem.* **2004**, *92*, 1193−1202.
(32) Janjetovic, K.; Harhaji-Trajkovic, L.; Misirkic-Marjanovic, M.; Vucicevic, L.; Stevanovic, D.; Zogovic, N.; Sumarac-Dumanovic, M.; Micic, D.; Trajkovic, V. *Eur. J. Pharmacol.* **2011**, *668*, 373−382.