DAW22, a natural sesquiterpene coumarin isolated from *Ferula ferulaeoides* (Steud.) Korov. that induces C6 glioma cell apoptosis and endoplasmic reticulum (ER) stress

Lan Zhang,a Xupeng Tong,b Jin Zhang,a Jian Huang,a,⁎ Jinhuì Wang,a,c,**

a Key Laboratory of Structure-Based Drug Design & Discovery of Ministry of Education, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016, China
b School of Pharmacy, China Pharmaceutical University, Nanjing 211198, China
c School of Pharmacy, Shihezi University, Shihezi 832002, China

**ARTICLE INFO**

Article history:
Received 25 February 2015
Accepted in revised form 8 March 2015
Accepted 9 March 2015
Available online 14 March 2015

Keywords:
*Ferula ferulaeoides* (Steud.) Korov.
DAW22
Apoptosis
C6 glioma cell
Endoplasmic reticulum (ER) stress

2,3-Dihydro-7-hydroxy-2R*,3R*-dimethyl-2-[4,8-dimethyl-3(E),7-nonenadienyl]-furo[3,2-c]coumarin (named DAW22), a sesquiterpene coumarin isolated from the roots of *Ferula ferulaeoides* (Steud.) Korov., has been reported to bear anti-proliferative activities toward different types of cancer cells. In this study, we demonstrated that DAW22 induced apoptosis in C6 glioma cells. Subsequently, we found that DAW22-induced apoptosis in C6 glioma cells occurred via the mitochondria-mediated and death-receptor pathways. Moreover, we found a massive cytoplasmic vacuolization, a dramatic change of endoplasmic reticulum (ER), up-regulation of CHOP and cleavage of caspase-12, suggesting that DAW22-induced apoptosis is involved in ER stress. In addition, we revealed that DAW22 treatment induced the activation of PERK, ATF6α and IRE1α. We further found that knockdown of CHOP affected DAW22-induced apoptosis, and DAW22-stimulated down-regulation of Bcl-2, caspase-8 activation and PARP cleavage were inhibited. Taken together, these results demonstrate that DAW22 induces apoptosis by ER stress and mitochondrial/death-receptor pathways, which may provide a new clue for exploiting this compound as a potential anti-neoplastic drug in future glioma cancer therapy.

© 2015 Elsevier B.V. All rights reserved.

**ABSTRACT**

1. Introduction

Glioblastoma is the most prevalent brain tumor in adults, with a median survival of approximately one year [1]. Patients suffering from glioblastomas usually have poor prognosis which may due to the low efficiency of targeting their inherent apoptosis-resistant phenotype. Moreover, glioblastomas generally prove refractory to treatment by conventional chemotherapy [2]. Apoptosis, type I programmed cell death (PCD), leads to multiple morphological characteristics, including cell shrinkage, membrane blebbing and formation of apoptotic bodies (with or without nuclear debris) that are phagocytosed by surrounding cells or by phagocytes [3,4]. Apoptosis is mainly initiated by two classic pathways: the death receptor-related extrinsic pathway and the mitochondrial-mediated intrinsic pathway [5]. The extrinsic pathway is initiated by the ligation of transmembrane death receptors and then leads to the activation of caspase effectors; for instance, activated Fas binds to Fas-associated death domain (FADD) and then activates caspase-8 and downstream caspase-3 [4]. The intrinsic pathway is associated

http://dx.doi.org/10.1016/j.fitote.2015.03.010

©2015 Elsevier B.V. All rights reserved.
with the integrity of mitochondrial membrane that is regulated by Bax and Bcl-2. The decrease of the Bcl-2/Bax ratio results in the disruption of the mitochondrial outer membrane. Then, cytochrome c is released from the mitochondria to the cytosol to form the apoptosome with Apaf-1 for caspase-9 activation, thereby promoting the activation of caspase-3, leading to apoptosis [6,7]. Previous studies pointed out the ER as a third subcellular compartment implicated in apoptotic execution. Like mitochondria, ER is a repository for both pro-apoptotic and anti-apoptotic proteins [8].

Endoplasmic reticulum (ER) is a multi-functional organelle required for protein folding and processing. A number of physiological and pathological conditions can disturb the folding of proteins in the ER, leading to ER stress [9]. ER stress activates several pathways to eliminate damage; these pathways are termed unfolded protein response (UPR). The UPR signal has the dual-function of impairing the damage associated with ER stress or inducing cell death through apoptosis [10,11]. Under ER stress conditions, accumulating unfolded proteins lead to dissociation with GRP78 and activates the three ER transmembrane receptors: PERK, ATF6, and IRE1 [12]. Dissociation of GRP78 from PERK leads to its homodimerization and autophosphorylation and induces eIF2α phosphorylation. Phosphorylated eIF2α selectively up-regulates the translation of ATF4 and subsequently increases the expression of the transcription factor CHOP [13]. Active IRE1 leads to an unconventional splicing of the transcription factor XBP1 triggering the removal of 26 nucleotides and the translation of an active transcription factor. A particularly important target of IRE1α is the mRNA encoding X-box binding protein 1 (XBP1). Active IRE1α splices a 26 base pair region from the mRNA encoding X-box binding protein 1 (XBP1), resulting in an active XBP1 transcription factor (XBP-1s) which has a diverse range of target genes [10]. ATF6 translocates to the Golgi where it is cleaved into 2 fragments. The active fragment of ATF6 relocates to the nucleus where it up-regulates many ER-stress induced genes [14]. Prolonged ER stress results in transcriptional induction of CHOP, which regulates genes that participate in the apoptotic pathway [15,16]. Caspase-12 serves as a marker of ER stress-induced apoptosis, which is activated by ER stress, but apparently not by death receptor-mediated or mitochondrial-targeted apoptotic signals [17]. ER stress has recently received much attention as a molecular pathway that can be modulated to cause cytotoxicity in glioma cells, which may be a possible target to develop chemotherapeutic drugs of glioblastoma [18].

In our previous study, we isolated 16 compounds from the roots of Ferula fulvulaceoides (Steud.) Korov. The anti-proliferative activity of all these compounds was evaluated on C6, MCF-7 and HepG2 cells. Compound 7 (named DAW22), displayed the best activity on C6 cells [19]. DAW22, a sesquiterpene coumarin derivative was first reported by Isaka, et al. in 2001 [20]. However, there is no related report about the mechanisms of DAW22-induced cell death in cancer cells. Thus, our study was designed to evaluate the in vitro anti-proliferative activity of DAW22, and the mechanisms of DAW22-induced cell death in C6 glioma cells. The detailed mechanisms, if elucidated, may provide guidance as to how DAW22 can be a candidate drug used for future cancer therapy.

2. Experimental

2.1. Purification of DAW22 from Ferula fulvulaceoides (Steud.) Korov.

DAW22 was isolated from the roots of Ferula fulvulaceoides (Steud.) Korov. in accordance with our previous methods [19]. The structure of DAW22 was then identified by spectroscopic data and physico-chemical methods with purity higher than 95%. Purified DAW22 was stocked as 20 mM in DMSO.

2.2. Chemicals and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rhodamine 123, and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Z-VAD-FMK and ECL were purchased from Beyotime (Beijing, China). Antibodies against Bax, Bcl-2, FADD, caspase-3, caspase-8, caspase-9, caspase-12, p-PERK(Thr981), ATF6α, β-actin and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PARP, Bcl-xl, Bid, GRP78, CHOP, p-eIF2α[ser51], IRE1α, and XBP-1s were purchased from Cell Signaling Technology (CST, Beverly, MA).

2.3. Cell culture

C6 glioma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium–high glucose medium (GIBCO, NY, USA) supplemented with 10% fetal calf serum (FCS) (Shengma Yuanheng, Beijing, China), 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.03% L-glutamine and maintained at 37 °C with 5% CO₂ in a humidified atmosphere.

2.4. MTT assay

The inhibitory effects of DAW22 on C6 glioma cell viability was measured by MTT assay. The cells were suspended at a density of 5.0 × 10^4/ml and added to 96-well flat bottom microtiter plates at 100 μl per well. After incubation for 24 h, the cells were treated with different concentrations of the drug. After incubation for the indicated time, cell growth was measured by MTT assay. If required, the pan-caspase inhibitor Z-VAD-FMK (5 μM) was added to C6 cells 2 h before DAW22 treatment. The percentage of inhibitory ratio was calculated as follows:

\[
\text{Inhibitory ratio(%) } = \left( \frac{A_{490 \text{, control}} - A_{490 \text{, sample}}}{A_{490 \text{, control}} - A_{490 \text{, blank}}} \right) \times 100.
\]

2.5. Hoechst 33258 and Rhodamine 123 staining

After incubation with DAW22 for 24 h, the cells were stained with Hoechst 33258 or Rhodamine 123 at 37 °C for 30 min, and then the morphology was observed by fluorescence microscopy (Olympus, Tokyo, Japan).
2.6. Annexin-V/propidium iodide (PI) dual staining assay

Annexin V/PI dual staining assay was employed to determine the involvement of apoptosis in DAW22-induced cell death, using Annexin-V-FITC Staining Kit (Roche) according to the manufacturer's instructions. The pan-caspase inhibitor Z-VAD-FMK (5 μM) was added to C6 cells 2 h before DAW22 treatment.

2.7. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by a membrane-permeable cationic fluorescent dye (Rhodamine 123). After incubation with DAW22 for the indicated time, the cells were incubated with Rhodamine 123 in the dark at 37 °C for 30 min, then harvested and suspended in 400 μl PBS. The samples were subsequently analysed by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

2.8. Western blot analysis

The C6 glioma cells were harvested, washed twice with cold PBS and then lysed in whole cell lysis buffer, supplemented with 100 μg/ml of proteinase inhibitors at 4 °C for 1 h. After centrifugation (12,000 g at 4 °C for 10 min), the protein concentration was determined by a BCA Protein Assay Kit (CWBio, Beijing, China). Equal amounts of total proteins were separated by 12% SDS-PAGE, and transferred onto Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, incubated with indicated primary antibodies at 4 °C overnight and horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h, then visualized by using ECL reagents.

2.9. Transmission electron microscopy

The C6 glioma cells were treated with DAW22 for indicated time periods. The collected cells were fixed with 2.5% glutaraldehyde, then treatment was performed as previously described [21].

2.10. Transfection of siRNA

CHOP (si-CHOP) and negative control (si-NC) siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with 50 nM si-CHOP, or si-NC using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were used for subsequent experiments 24 h later.

2.11. Target prediction and molecular docking

The structure of DAW22 was downloaded from the latest version of DrugBank (http://www.drugbank.ca/) to construct the screening of its potential targets. Target proteins of the small molecule compounds were obtained from the Similarity ensemble approach (SEA) (http://sea.bkslab.org/) [22], which uses the similarity ensemble approach relating proteins based on the set-wise chemical similarity among their ligands. The initial three dimensional geometric coordinates of the X-ray crystal structure of ERG7 (PDB code: 1W6K) and DYRK1A (PDB code: 3ANQ) were downloaded from the Protein Data Bank (PDB) (http://www.pdb.org/pdb/home/home.do). In addition, we used Accelrys Discovery Studio version 3.5 (Accelrys Inc., USA) with CHARMM force-field parameters to dock pre-generated conformations of DAW22 into its targets for testing the binding conformation of the complex. We performed flexible-ligand docking to a rigid receptor with grid-based scoring, in which DAW22 was allowed to be flexible and structurally rearranged in response to its targets. The pathways of apoptosis and ER stress were obtained according to previous reports [23–30].

2.12. Statistical analysis

All the presented data were confirmed in at least three independent experiments and are expressed as the mean ± SD. Statistical comparisons were made by Student's t-test. p < 0.05 was considered statistically significant.

3. Results and discussion

3.1. DAW22 inhibits cell growth and induces apoptosis in C6 glioma cells

The C6 glioma cells were treated with different concentrations of DAW22 (6.25, 12.5, 25, 50, and 100 μM) for 24, 36, and 48 h and cell viability was determined by MTT assay. Our results showed that DAW22 inhibited C6 glioma cell growth in a time- and concentration-dependent manner with an IC50 value (at 24 h) of 18.92 μM (Fig. 1B). In order to determine features of reduced cell viability induced by DAW22, the morphologic changes of cells were examined by phase-contrast microscopy. Compared with the control group, treatment of DAW22 caused remarkable morphologic changes such as membrane blebbing and apoptotic bodies (Fig. 1C). These changes were further confirmed by Hoechst 33258 staining. The cells in the control group showed uniform dispersion of low-density fluorescence, but DAW22 treated cells showed condensed, bright fluorescence and nuclear fragmentation (Fig. 1D). Then the ultrastructure of cells was examined by transmission electron microscopy. The C6 glioma cells treated with DAW22 showed typical characteristics of apoptosis: cytoplasmic vacuolization, condensation and margination of nuclear chromatin (Fig. 1E). Thus, these results suggest that DAW22 induces apoptosis in C6 glioma cells.

3.2. Caspase activation involves in DAW22-induced apoptosis

We firstly found that DAW22 induced the cleavage of PARP (Fig. 2A), suggesting caspase activation, which is a significant feature of cell apoptosis. Then, we examined whether DAW22 induces caspase activation. Caspase-3, caspase-8, and caspase-9 were detected by Western blot analysis. The results revealed a decrease of procaspase-3, 8, and 9 and a marked increase of cleaved caspase-3, 8, and 9, indicating that these caspases were activated (Fig. 2A). To further confirm whether DAW22-induced cell death was caspase-dependent, the cells were pretreated with Z-VAD-FMK (pan-caspase inhibitor, 5 μM) before treatment with DAW22. Our results showed that Z-VAD-FMK treatment suppressed the cell death induced by
DAW22. The level of DAW22-induced cell death was decreased from 46.61 ± 0.31% to 26.70 ± 2.80% when co-incubated with Z-VAD-FMK (p < 0.05) (Fig. 2B). Moreover, flow cytometric analysis with Annexin V/PI demonstrated that Z-VAD-FMK treatment suppressed the level of apoptotic cells upon DAW22 stimulation. The late apoptotic cell ratio was reduced from 26.83 ± 1.20% to 10.47 ± 0.81% when co-treated with Z-VAD-FMK (p < 0.001) (Fig. 2C). These results indicated that DAW22-induced apoptosis was partially caspase-dependent.

3.3. DAW22-induced apoptosis occurs in both intrinsic and extrinsic pathways

The activation of caspase-9 and caspase-8 suggested that DAW22 induced apoptosis may be through the mitochondrial-mediated intrinsic pathway and death receptor-mediated extrinsic pathway. The down-regulation of the Bcl-2/Bax protein ratio can disrupt the mitochondrial membrane potential (MMP). Therefore, we first examined the integrity of the mitochondrial membranes of the cells with Rhodamine 123 staining by a fluorescence microscope and flow cytometric analysis. DAW22 treatment resulted in a decrease of MMP in a time-dependent manner (Fig. 3A and B). Next, several proteins of the Bcl-2 family such as Bax, Bcl-2, and Bcl-xL and the death receptor-related protein Fas and FADD were detected by western blot analysis. The results demonstrated that the expression of Bax elevated markedly, while the protein level of Bcl-2 and Bcl-xL decreased (Fig. 3C). In addition, the protein expression level of Fas and FADD was apparently increased. We also found that DAW22 treatment stimulated Bid cleavage, which was a downstream protein of caspase-8 (Fig. 3D). Thus, DAW22-induced apoptosis is via both intrinsic and extrinsic pathways.

3.4. DAW22 induces apoptosis via ER-stress and unfolded protein response

Massive cytoplasmic vacuolization was observed in the cells with DAW22 treatment for 24 h (Fig. 4A). Therefore, we examined the ultrastructure of DAW22 treated cells by transmission electron microscopy. The control group showed a normal cell phenotype. Compared with the control cells, the
DAW22 treated cells displayed several apoptotic features such as chromatin condensation, nuclear fragmentation, and cytoplasmic hypervacuolization; especially longer treatment resulted in more and bigger vacuoles (Fig. 4B). In addition, DAW22 induced cytoplasmic vacuolization was not due to autophagy because DAW22 did not induce autophagosome formation in the cells. It was conceivable that DAW22-induced ER morphological change may reflect ER-stress and thus ER dysfunction. Thus, we examined whether ER-stress was induced by DAW22 via detecting the expression levels of GRP78, CHOP and caspase-12. Our data indicated that caspase-12 was activated and the expression of GRP78 and CHOP was up-regulated with DAW22 treatment in a time-dependent manner (Fig. 4C). ER-stress might trigger UPR through the activation of signaling proteins including PERK, IRE1, and ATF6. Therefore, we next monitored the effects of DAW22 on three signaling pathways of UPR by immunoblotting. We found that PERK was auto-phosphorylated at Thr981 upon DAW22 treatment. Phosphorylation of eIF2α was detected in cells treated with DAW22 for 6 h but the phosphorylated eIF2α protein level slightly decreased after 6 h. This may be due to the dephosphorylation of eIF2α caused by increasing apoptosis [31]. ATF6α decreased steadily from 6 h to 24 h of DAW22 treatment, manifesting that this protein was cleaved into the activated form. The up-regulation of IRE1α and the activation of XBP-1s (splicing form of XBP1) indicated that the IRE1 branch of UPR signaling was activated (Fig. 4D). CHOP protein is a pro-apoptotic molecule through regulating the mitochondrial and death receptor pathways. To further explore whether CHOP activation was associated with extrinsic and intrinsic pathways, we examined the expressions of caspase-8 and Bcl-2 in control siRNA and CHOP siRNA-transfected cells. Our data showed that CHOP was markedly decreased by the specific CHOP siRNA. Also, the silence of CHOP expression significantly reduced caspase-8 activation and restored Bcl-2 expression. Moreover, we also found that inhibition of CHOP blocked DAW22-induced PARP cleavage (Fig. 4E). Taken together, these results demonstrate a critical regulatory role for CHOP in DAW22-induced apoptosis.

3.5. DAW22 target prediction and its possible mechanisms of apoptosis and ER stress

To further identify the mechanism of DAW22 in cell apoptosis and ER stress, we predicted the targets of DAW22 and their relevant pathways computationally. ERG1, ERG7 and
DYRK1A, as targets of DAW22 predicted by SEA, indeed regulated cell apoptosis and ER stress. The DAW22 binding conformations in the complexes with ERG7 or DYRK1A were calculated (Fig. 5A). Secondly, the pathways of apoptosis and ER stress were obtained according to previous reports (Fig. 5B). The inhibition of ERG1 and ERG7 induced the depletion of cholesterol, which further induced ER stress. Also, the changed expression level of proteins in an unfolded protein response induced cell apoptosis through the pathways, which was experimentally verified as mentioned above. Moreover, DAW22 may inhibit DYRK1A to induce cancer cell apoptosis.

3.6. Discussion

In the last few decades, small molecules of plant origin became valuable sources of potential lead compounds in anti-cancer drug discovery [32]. DAW22, a natural active compound isolated from Ferula ferulaeoides (Stud.) Korov. was found to have in vitro anti-proliferative activities against several tumor cell lines. In this study, we demonstrated for the first time that DAW22 inhibited cell growth on C6 glioma cells in a time- and concentration-dependent manner, and induced apoptosis. Our results showed that DAW22-induced apoptosis was executed through ER-stress, the mitochondrial and death receptor pathways.

In recent years, a number of anti-cancer therapies have been associated with the induction of ER stress in tumor cells. It has been reported that ER stress induced apoptosis and autophagy in human glioma cells [33]. However, the researches of apoptosis on C6 glioma cells were commonly focused on cell cycle arrest, mitochondrial pathway and reactive oxygen species (ROS) generation [34–36]. Some biologically active sesquiterpene coumarins from the Ferula species such as ferulenol and umbelliprenin have not been reported as being related to ER stress [37,38]. But in our study, we found that DAW22 induced phosphorylation of PERK and eIF2α, cleavage of ATF6α, up-regulation of IRE1α and expression of XBP-1s. Moreover, DAW22 also elevated the protein level of GRP78, CHOP and cleavage of caspase-12. These findings suggest that DAW22 induces apoptosis via ER stress and activates all three UPR branches in C6 glioma cells.

Fig. 3. DAW22-induced apoptosis occurs in both intrinsic and extrinsic pathways. A) The cells were treated with DAW22 (20 μM) for 24 h then stained with Rhodamine 123 and observed by fluorescence microscope (×200 magnification). Bar = 20 μm. B) The cells were treated with DAW22 (20 μM) for 6, 12, and 24 h. MMP was then detected by flow cytometry staining with Rhodamine 123. C) The cells were incubated with DAW22 (20 μM) for 6, 12, and 24 h, followed by western blot analysis for the detection of Bax, Bcl-2 and Bcl-xL levels. D) The cells were treated with DAW22 (20 μM) for 6, 12, and 24 h, followed by immunoblotting to detect the levels of Fas and FADD. β-Actin was used as an equal loading control.
Previous studies have suggested that ER stress-induced apoptosis is associated with mitochondrial and death receptor pathways [39]. According to our results, DAW22 could induce both mitochondrial and death receptor pathways which involved down-regulation of the Bcl-2/Bax ratio, reduced expression of Bcl-xL, loss of mitochondrial membrane potential, activation of caspase-9, up-regulation of Fas and FADD protein, cleavage of Bid and activation of caspase-8. We observed that the knockdown of CHOP expression by siRNA significantly inhibited the cleavage of PARP and caspase-8 activation. It also up-regulated the level of the Bcl-2 protein decreased by DAW22. These results indicated that CHOP plays a role in the regulation of these pathways.
an important role in ER stress-induced apoptosis on DAW22 treated cells. Interestingly, in silico analysis further demonstrated that DAW22 targeted DYRK1A and ERG7 that might result in the regulation of ER stress and apoptosis of C6 glioma cells. These results would shed new light on providing more novel targets for DAW22 and its possible signaling pathways in our future studies.

In conclusion, our results indicate that DAW22, a sesquiterpene coumarin isolated from the roots of Ferula ferulaeoides (Steud.) Korov. is a promising anti-proliferative natural compound, which can inhibit C6 glioma cell growth by death-receptor and mitochondrial apoptosis and ER stress. Therefore, these findings would provide a clue for exploiting this compound as a potentially novel anti-neoplastic drug in glioma cancer therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We are grateful to Prof. Bo Liu and Liang Ouyang (Sichuan University) for revising our English. This work was supported by the Key Projects of the National Science and Technology Pillar Program (No. 2012BAI30B02), the National Natural Science Foundation of China (Nos. U1170302, and 81303270) and the Shenyang Pharmaceutical University Scientific Research Fund (ZCJJ2013407).

References


Pan L, Chai HB, Kinghorn AD. Discovery of new antitumor agents from higher plants. Front Biosci (Schol Ed) 2012;4:142–56.


