The cell toxicity effect of secalonic acid D on GH3 cells and the related mechanisms

GUIZHI LIAO1,4, JING ZHOU1, HUI WANG1, ZHIGANG MAO2, WEIWEI XIAO1, HAIJUN WANG2, ZHIGANG SHE3 and YONGHONG ZHU1

1Department of Histology and Embryology, 2Department of Neurosurgery, the First Affiliated Hospital, 3Department of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou, Guangdong 510080, 4Department of Neurosurgery, Guangzhou General Hospital of Guangzhou Military Command, Guangzhou 510010, P.R. China

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Correspondence to: Dr Yonghong Zhu, Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China
E-mail: zhuyongh@mail.sysu.edu.cn

Dr Zhigang She, Department of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China
E-mail: cesszhzhg@mail.sysu.edu.cn

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Abstract. To investigate the anti-pituitary adenoma effect of secalonic acid D (SAD) extracting from marine microorganisms, we used MTT colorimetric method to evaluate the proliferation of GH3 cells treated by SAD, the time- and dose-dependent effects and the value of IC50 were recorded. Hoechst staining, TUNEL and flow cytometry methods were used to analyze the apoptosis rate of GH3 cells treated by SAD. Western blotting, RT-PCR and caspase inhibitor (Z-VAD-FMK) were used to investigate the possible mechanism of SAD induced apoptosis and the expression of growth hormone (GH). The results showed that SAD has a time- and dose-dependent effect on GH3 cells and the cytotoxic effect was mainly through apoptosis. The mechanisms were partly through the activity of caspase family and also G1/S phase block. In addition, SAD also can suppress the expression levels of growth hormone in GH3 cells, however, the RT-PCR results showed that the mechanism was not through changing the expression of GH mRNA. We concluded that SAD may be a potential anti-pituitary tumor drug and further in vivo studies should be performed.

Introduction

The pituitary gland located in Hypophyseal Fossa is composed of neurohypophysis and adenohypophysis. It is a central endocrine organ that have important functions in maintaining homeostasis, controlling hormone recycle, and activating the other gland organs. It can regulate the systems of growth and metabolism cooperated with the nervous system (1). A mutation on adenohypophysis always induces several important systems dysplasia or dysfunction through parasecretion or suppressing surrounding tissues. Patients suffer a very poor life quality with pituitary adenoma and the final diagnosis and rational therapy are quite difficult because of the different differentiation states of tumor cells. Pituitary adenoma always occur in young adults and have severe influence on growth development, daily work and fertility, unsuitable diagnosis and treatment will bring a great burden on the patients, family and the society.

According to studies from the US and Italy, clinically diagnosed pituitary adenoma occur with a prevalence of 19-28 cases/100,000 population (4), being 10% of the central nervous system tumors (2-4). Clinical diagnosis is established 5-10 years after the development of a pituitary adenoma, because most of the tumors are benign and grow very slowly and no obvious symptoms exist. Ezzat et al (5) undertook a systematic review of all autopsy and radiological studies of the prevalence of pituiray adenomas, they found that the mean prevalence was ~16.7% or one in six individuals. Such a high morbidity attracted more research attention. Surgical resection is the main treatment of pituitary adenoma, while drug therapy was an important adjunctive treatment. With the development of pathogenesis research and new drugs, the position of pharmacotherapy has became increasingly important. Some therapies efficiently suppress volume of the tumors and the hyper-secreting hormone, such as bromocriptine, somatropin analogue (6,7). However, Chanson et al (8) pointed out that there are hardly any effective drugs for non-functioning pituitary adenomas (NFAs), while Somatostatin analogues are effectively only on GH adenoma expressing SSTR2 or SSTR5 and no obvious effect on other subtypes (9,10).

Marine endophytic fungi have been proven to be a rich source of new biologically active natural products for they present a relatively untapped ecological environment, and their secondary metabolism molecules was particularly active because of the interactions with their hosts. Remarkably, many of these biologically active molecules have potential utility in modern medicine and biomedical research. Marine
natural products, which were isolated from sponges, microorganisms and tunicates are currently in clinical or pre-clinical trials for the treatment of tumors (11,12). However, few researchers have reported on the medical treatment of pituitary adenoma from marine products. We report on an effective marine fungi compound for pituitary adenoma cells. The compound used in our experiment is a marine natural product extracted from Paecilomyces sp. (tree 1-7) and SBE-14 of South China Sea (13), and we found that the compound has the same struture as secalonic acid D (SAD) (Fig. 1). Although there are studies on the antitumor effects such as leukemia (14), bladder carcinoma (15), no research on pituitary adenoma was found. We found a significant supression of GH3 cells (rat pituitary adenoma cell line) on cell proliferation and GH expression treated by SAD.

Materials and methods

Chemicals and reagents. SAD was provided from the Department of Chemistry and Chemical Engineering of Sun Yat-sen University (16). GH3 cell line was purchased from ATCC (USA). Anti-rat GH antibody and anti-rat β-actin antibody were purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Invtrogen Biotechnology. TUNEL kit purchased from Roche Biotechnology.

Cell culture. The rat pituitary adenoma cell line GH3 cells were cultured in complete medium (F-10 supplemented with 15% horse serum and 2.5% FBS) as previous described (17). Complete medium was changed every 2-3 days, and transfer of culture (1:2-3) every 6-7 days by 0.25% trypsogen-0.02% EDTA. The cells were plated at a density of 1x10⁶/well in a 6-well plate for Western blotting, 1x10⁵/well in a 24-well plate for radioimmunological kit, 2x10⁴/well in a 96-well plate for cell proliferation experiment.

MTT assay. The cell proliferation of SAD treated on GH3 cells were measured by MTT assay. Briefly, GH3 cells were seeded on 96-well plates (Corning, USA) in the presence of various concentration of SAD (10⁻⁸-10⁻⁴ M) for 48 h as a pre-experiment. Then, different concentration of SAD (1.25x10⁻⁶, 2.5x10⁻⁶, 5x10⁻⁶, 10x10⁻⁶, 20x10⁻⁶, 40x10⁻⁶ M) were treated on GH3 cells in a final volume of 100 μl for indicated times. Thereafter, 10 μl MTT (5 g/l) was added to each well and incubated for 2 h, 100 μl developer solution (SDS w/v 20%; isopropanol v/v 10%; 1 M hydrochloric acid v/v 2%) was added and incubated overnight at 37°C. Optical density was determined at 570 nm by using a microplate reader. The following formula was used: cell proliferation inhibited (%) = [1 - (OD of the experimental samples / OD of the control)] x100%; IgIC₅₀=Xm-I(P-(3-Pm-Pn)/4; Xm, maximal dose; I, maximal dose/adjacent dose; P, sum of positive reaction rate; Pm, maximal positive reaction rate; Pn, minimal positive reaction rate.

Flow cytometric analysis of cell cycle and apoptosis. Cell cycle distribution and apoptosis was determined by staining DNA with propidium iodide (PI). Briefly untreated or SAD treated cells (0.3 μM for 12, 24, 48 h) were centrifuged, washed with PBS and fixed in 70% pre-cooled ethanol. The tubes containing the fixed cells were stored at 4°C for at least 24 h. After this, the cells were centrifuged at 1,500 rpm for 5 min, and the supernatant was discarded to remove ethanol completely. The cells were washed by PBS and stained with PI solution [100 μg/ml PI from 50X stock solution (2.5 mg/ml), 0.1 mg/ml RNase A, 0.05% Triton X-100], incubated in the dark for 40 min at 37°C. After 3 ml PBS was added, the cells were pellet (1,500 rpm, 5 min) and the supernatant was taken off. Then the pellet was resuspended with 300 μl PBS for flow cytometric analysis. Cells with DNA content below G1 phase (peak of hypodiploid DNA below G1 phase) were regarded as apoptotic cells. The cells in G0/G1 phase, S phase and G2/M phase were analyzed by muticycle software.

Hoechst 33342 staining. Apoptotic bodies were observed by Hoechst 33342 staining. Briefly, SAD (IC₅₀) treated or untreated cell climbing slides were fixed in 4% paraformaldehyde at room temperature for 1 h. Then the slides were washed with 3X PBS for 5 min, and incubated with Hoechst 33342 (50 μg/ml) for 5-10 min in dark. Washed with 3X PBS for 5 min and the apoptotic bodies were observed under a fluorescence microscope.

TUNEL assay. The TUNEL methods was used as previously described (18) to assess the DNA fragmentation. Briefly, GH3 cells treated by SAD for different times were fixed for 1 h in 4% paraformaldehyde at room temperature. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide in methanol for 30 min at room temperature and further incubated in a permeabilizing solution (0.1% Triton X-100) for 2 min at 4°C. The cells were incubated with the TUNEL reaction mixture for 60 min at 37°C, followed by labeling with peroxidase conjugated anti-fluorescein anti-sheep antibody Fab fragments for additional 30 min. TUNEL-positive cells were green under fluorescence microscope and brown with DAB (3,3′-diaminobenzidine tetrahydrochloride) staining. The apoptotic rate of SAD-treated cells was calculated by the formula: (apoptotic rate = TUNEL-positive cells/(TUNEL-positive cells + TUNEL-negative cells) x100%.

RT-PCR analysis for expression of GH mRNA. Total RNA of GH3 cells treated by SAD for indicate times were extracted using TRIzol reagent (Takara Bio-Tek, Dalian, China). Briefly, the RT-PCR method was used as previously described (19). For RT, the SuperScript first-strand synthesis system kit
(Invitrogen) was used. RT products (cDNA) (2 μl) were amplified with GH primers as follows: GH sense primer: 5′-ACTCCCTGGCTCCTGACCTT-3′, GH antisense primer: 5′-GGATGAGCAGCAGCGAGAA-3′. These primers were designed by Primer Premier 5.0 software. The predicted product is 307 bp. Amplification was carried out as follows: 1x (10 min at 94˚C), 35x (45 sec at 94˚C, 45 sec at 56.5˚C, and 45 sec at 72˚C), and 1x (5 min at 72˚C). A portion (5 μl) of RT-PCR product was electrophoresed in 1% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed using Bio-imaging system (Sygene Genegenius, USA).

**Western blot analysis.** Aliquots (60 μg of protein) of cell lysates were separated on 12% SDS-PAGE, blotted onto a PVDF membrane (Bio-Rad, USA). Membranes were washed twice with 0.05% Tween-20 in TBS (pH 7.6) and then incubated with blocking buffer (5% non-fat milk in TBST) for 60 min. Overnight incubation of membranes with primary antibodies (anti-rat GH 1:1000, anti-rat β-actin 1:2000) were done at 4˚C, then washed 3 times with TBST. Membranes were incubated with secondary antibodies at room temperature for 60 min, washed 3 times with TBST and antibody bound proteins were detected by enhanced chemiluminescence reagents (Pierce).

**Caspase inhibition assay.** Whether SAD induced GH3 cell apoptosis through caspase pathway was further measured using an MTT based assay. Briefly, the cells (10000/well) were incubated in a 96-well plate. Caspase inhibitor Z-VAD-FMK (20 μM) was added 15 min before SAD (IC50) in a final volume of 100 μl and incubated for different times (12, 24, 36, 48 h). One control was the absence of caspase inhibitor and the other was absence of SAD and the caspase inhibitor. Then the inhibition effects were measured by MTT analysis.

**Statistical analysis.** Data are presented as the mean ± SD per group. Statistical analysis was performed using Student t-test and ANOVA followed by multiple comparison tests. *P*<0.05 was considered to be statistically significant. Experiments, both the treated group and the control, were carried out in triplicate.

**Results**

*SAD inhibits pituitary adenoma cell proliferation in a dose-dependent manner.* We investigated the effect of SAD on GH3 cell proliferation. GH3 cells were treated with SAD in a dose-dependent manner (0.125-4.0 μM) and cell proliferation was assessed by MTT assay after incubated for 48 h. After treatment with SAD (IC50) for 48 h, the morphology was changed (Fig. 2A and B). The cells shrunk, got smaller,
broke into smaller pieces, and most of the cells were floating compared with the control. The results of MTT assay showed that SAD, in a dose-dependent manner inhibited GH3 cell proliferation (Fig. 3A). With the rise of SAD concentration, the cell survival rate decreased gradually. When the cells were treated with 4 μM for 48 h, only 20% survived compared with the control, however, when the concentration was diluted to 0.125 μM, there was hardly any toxic effects on GH3 cells. We calculated the 50% inhibition concentration (IC50) as 0.357±0.083 μM.

Figure 3. GH3 cells were treated with indicated concentration of SAD (4.0, 2.0, 1.0, 0.5, 0.25, 0.125 μM). When the dilution range of SAD was 0.25-4.0 μM, the proliferation was in a dose-dependent manner decreased (A). (B) Three different concentrations of SAD (0.2, 0.4, 0.6 μM) were treated on GH3 cells for 6 days and cell proliferation was assessed by MTT method, (*P<0.05).

Figure 4. The cell cycle phase were observed on GH3 cells treated by SAD (0.3 μM) for 0, 12, 24, 48 h. The results indicate that with longer incubating time, the percent of G1 phase increased and S phase decreased compared with the control group (0 h).
SAD inhibits pituitary adenoma cell proliferation in a time-dependent manner. We next determined the time-dependent manner treated with SAD for 6 days by MTT assay (Fig. 3B). The results indicated that SAD (0.6 μM) incubated for 2 days can induced ~70% GH3 cells death, and after incubated for 4 days, nearly all cells died; SAD (0.4 μM) incubated for 2 days can induced ~60% GH3 cells death, and after incubated for 5 days, nearly all cells died; SAD (0.2 μM) incubated for 2 days can induce ~30% GH3 cell death, the maximal cell death rate (80%) was achieved for 6-day incubation, and no significant changes after that (data not shown). We chose 0.3 μM SAD incubated for 48 h as the best concentration and treatment time for the later experiments according to the IC50 and time-dependent experiments.

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SAD induces pituitary adenoma cell changes of cell cycle and apoptosis in a time-dependent manner. We observed the GH3 cell cycle changes and the cell apoptosis rate treated with SAD through flow cytometric (FCM) analysis. After treatment with SAD (0.3 μM) for 12, 24, 48 h, the number of G0/G1 and G2/M phase in GH3 cells were significantly increased and decreased, respectively (Fig. 4). The cell proliferation inhibition likely blocked the cell cycle progression from G1 to S phase. The analysis of DNA contents by FCM showed the number of hypodiploid DNA (point to apoptosis) before G1 phase in GH3 cells treated by SAD for 12, 24, 48 h, and control group were 2.5, 8.6, 46.8, 2.6%, respectively (Fig. 5). The apoptosis rate was significantly increased in 24 h and 48 h groups, while no obviously changes in 12 h group compared with control group. The results showed that the SAD induced GH3 cells apoptosis in a time-dependent manner.

Hoechst staining and TUNEL assay were also used to investigate whether treatment by SAD causes apoptosis or necrosis and the apoptosis rate. Apoptotic bodies were observed in SAD treated group with Hoechst staining and no apoptotic bodies were found in the control group (Fig. 2C and D). Cells with condensed, fragmented nuclei was scored as apoptotic. The apoptotic nature of these cells was conformed by TUNEL staining, TUNEL-positive cells showed brown color nuclei, and the apoptotic rate was calculated (Fig. 6). The apoptotic rates were 7.72, 8.68, 20.65, 48.64%, respectively, after SAD treatment for 0, 12, 24, 48 h, for the 24 h and 48 h groups the apoptotic rates had significant differences compared with the control (Fig. 7). TUNEL assay results were consistent with the results obtained from FCM analysis. The above indicated that the cell toxicity effect of SAD-induced GH3 cell death was mainly through apoptosis.

SAD decreased growth hormone (GH) expression levels in GH3 cells, but not the GH mRNA levels. We investigated whether SAD had an influence on hormone expression in GH3 cells by Western blot analysis and RT-PCR methods. GH3 cells were incubated with SAD for 0, 12, 24, 48 h, and GH content in equal amount of cell lysates was determined by Western blotting. Our results show (Fig. 8A) that SAD in a time-dependent manner decreased the expression of GH in GH3 cells, the expression of GH decreased at 12 h, and nearly completely blocked GH expression after 48 h.
incubation. We also investigated whether the decreased expression of GH was through down-regulating mRNA level by RT-PCR, however, we found no significant changes of mRNA in GH3 treated with different concentrations (0, 1.0, 0.5, 0.25, 0.125, 0.65 μM) for 12 h (Fig. 8B). The results indicated that SAD can reduce GH protein expression through other than the mRNA level.

Caspase inhibitor Z-VAD-FMK can reduce the apoptosis rate induced by SAD. In order to explore whether the mechanism of SAD inducing GH3 cell apoptosis was through the caspase pathway. We added 20 μM caspase inhibitor Z-VAD-FMK to GH3 cells treated by SAD (IC_{50}) at different times and analyzed by MTT assay. The results showed that Z-VAD-FMK can partly inhibit SAD induced apoptosis at 36 h and 48 h (Fig. 9).

**Discussion**

Pituitary adenoma is a common benign tumor with several pathological effects due to excessive hormone secretion and tumor press the surrounding tissues. The incidence rate of pituitary adenoma is increasing, however, there are only two effective therapeutic drugs, Dopamine 2 (D2) agonists and a Somatostatin analogue. Moreover, it is reported that ~10-25% nonfunction pituitary adenoma (NFPA) have no effect treated by D2 agonists bromocriptine (8), and 5-10% patients have intolerance to the serious side-effects such as dizziness, nausea, and arrhythmia. Certain GH adenoma patients are also insensitive to Somatostatin analogue therapy (9,10). Miller *et al* (20) found that curcumin, a commonly used food additive can effectively inhibit pituitary adenoma cell proliferation, induce apoptosis and decrease hormone secretion. Cervia *et al* (21) found that euplotin C, isolated from the ciliate Euplotes crassus can induce Ca^{2+} stores in the endoplasmic reticulum (ER), release of cytochrome c from the mitochondria, activation of caspase-12, and activation of caspase-3, leading to apoptosis in pituitary adenoma ATt20 cells. In this study, we examined the role of SAD, a natural product extracted from marine fungi, that had a potential neurotoxicity effect on pituitary adenoma cells and induced cell apoptosis, moreover, SAD also can inhibit the expression level of GH in vitro.

Our data showed that SAD in a dose- and time-dependent manner inhibited the proliferation of pituitary adenoma GH3 cells by MTT assay. The inhibitory effect of SAD (0.3 μM) was apparent within 24 h of treatment, incubated for 48 h the inhibitory effect can achieve 50%, and persist up to 6 days SAD can nearly completely block the proliferation of GH3 cells and induced cell death. The half inhibitory concentration was calculated as 0.357±0.083 μM in our experiments, and we also compared the IC_{50} of many other marine compounds treated adenocarcinoma cells. Antipathine A, extracted from China Sea had a cell toxicity effect on gastric carcinoma (SGC-7901) cells, and the IC_{50} was 67.38 mg/ml (22); The IC_{50} of hantupeptin on leukemic cells (MOLT-4) and breast carcinoma cells (MCF-7) were 32 and 4.0 mM, respectively (23); the IC_{50} of kuanoniamine A and kuanoniamine C on MCF-7 cells were 0.12±0.07 μM and 0.81±0.11 μM, respectively (24). We concluded that SAD...
had a much stronger inhibition effect on GH3 cells compared to the other anti-adenocarcinoma compounds.

Whether the inhibitory effect is through apoptosis or necrosis was further investigated by observing the apoptosis rate induced by SAD. Considering that apoptosis plays a central role in regulation of pituitary tissue homeostasis, the imbalance between cell death and proliferation in favor of cell survival could result in tumor formation. One of the commonly employed strategies in clinical/experimental therapy for cancer is to trigger tumor cell apoptosis (25). Clinical chemotherapeutics, such as alkylating agents, cisplatin and paclitaxel, can suppress cell proliferation and induce tumor cell apoptosis (26). Therefore, whether SAD can induce GH3 cell apoptosis was a very important aspect for further research, and Hoechst 33342 staining, TUNEL assay, FCM were used to observe the apoptosis rate induced by SAD. The results showed that the cell toxicity effect of SAD was mainly through apoptosis, and in a time-dependent manner, therefore we believe that SAD may be an effective natural compound for promoting apoptosis. Moreover, the FCM also showed that SAD can block G1/S phase transition in the cell cycle reducing proliferation of GH3 cells, and initiating apoptosis.

It is clear that caspase family members play important roles in driving apoptosis. Activation of caspase appears for many of the molecular and structural changes in apoptosis. There also exists an active caspase-independent programmed cell death (PCD), such as apoptosis inducing factor (AIF) and Endonuclease G (27,28). SAD-induced apoptosis through caspase was determined by caspase inhibitor Z-VAD-FMK. The results indicated that blocking caspase activity can partly reduce the apoptosis rate, however, only ~20% cells survived in caspase inhibitor-treated group compared with the absence of caspase inhibitor group in different incubations. We concluded that the mechanism of SAD induced apoptosis was partly through the caspase pathway, but also through another pathway. AIF is believed to play a central role in the regulation of caspase-independent cell death and it is released from mitochondria in response to apoptotic stimuli (29).

Hyperprolactinemia and excessive levels of GH are commonly associated with pituitary adenoma. We questioned whether SAD had any effect on the levels of GH in GH3 cells. RT-PCR and Western blotting were used to investigate the changes of GH mRNA level and protein level, respectively. Finally, we demonstrated that SAD had a potent time-dependent inhibitory effect on GH expression in GH3 cells, but no significant changes were observed in GH mRNA incubated by different concentrations of SAD for 12 h. This result suggests that SAD can modify the post-translational

Figure 8. The expression of GH in GH3 cells treated with SAD for 0, 12, 24, 48 h was assessed by Western blotting. The result showed that the expression of GH was time-dependently decreased (A). The changes of GH mRNA treated with SAD for different concentrations at 12 h were assessed. The results indicated that no significant changes of GH mRNA was observed in GH3 cells treated with SAD compared to the control of β-actin (B).

Figure 9. Caspase inhibitor Z-VAD-FMK (20 μM) and STb (0.3 μM) were incubated with GH3 cells to assess the function of caspase family in the process of STb-induced cell apoptosis. The results indicated that caspase inhibitor can partly inhibit the death of GH3 cells treated by STb at 36 h and 48 h (*P<0.05).
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level and induces the reducing product of GH protein. Post-translational modification of important gene products have a close association with tumorigenesis, and could become a new therapeutic target for tumors. Casado et al (30) found that vincristine regulated the phosphorylation of the apoptotic protein HSP27 in breast cancer cells and induced cancer cell apoptosis. There are many drugs such as tetracycline (31), chloramphenicol (32), and erythromycin (32) that can inhibit the conjunction of aminoacyl-tRNA, transpeptidase and peptide formation. SAD may also affect organelles such as mitochondria, ribosome and endoplasmic reticulum, for pituitary adenoma cells have activity in synthesis and secretion of many kinds of hormones and abundance of organelles. It is reported that many important factors that regulate the expression of GH were synthesized and secreted in endoplasmic reticulum and Golgi such as Somatotropin release inhibiting factor (SRIF) (33), vascular endothelial growth factor (VEGF) (34), carboxypeptidase D (CPD) (35). SAD may indirectly influence these factors to reduce the expression of GH.

In conclusion, we have shown for the first time that SAD inhibits proliferation, and induces apoptosis. In addition we show that SAD decrease cell proliferation and apoptosis may be through cell cycle blocking, via caspase activation. We also demonstrated that SAD suppresses intracellular GH hormone levels, but has no influence on mRNA level of GH in the first 12 h while the expression of GH protein declined. Based on these data, we propose that SAD may be a novel therapeutic compound in the management of GH adenomas, but further study is required.