Soloxolone methyl, as a 18βH-glycyrrhetinic acid derivate, may result in endoplasmic reticulum stress to induce apoptosis in breast cancer cells

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ABSTRACT

Being one of the leading causes of cancer death among women, various chemotherapeutic agents isolated from natural compounds are used in breast cancer treatment and consequently studies to develop new drugs still continue. There are several studies on 18βH-glycyrrhetinic acid, a secondary metabolite which is found in Glycyrrhiza glabra (liquorice roots), as a potential anticancer agent. In this study, the cytotoxic and apoptotic effects of Soloxolone methyl compound, a semisynthetic derivative of 18βH-glycyrrhetinic acid were investigated on breast cancer cells (MCF-7, MDA-MBA-231). Soloxolone methyl is found to be cytotoxic on both MCF-7 and MDA-MBA-231 breast cancer cells by inducing apoptosis. Especially in MDA-MB-231 cells apoptosis is detected to be triggered by ER stress. The antigrowth effects of Soloxolone methyl were determined using MTT and ATP assays. To identify the mode of cell death (apoptosis/necrosis), fluorescent staining (Hoechst 33342 and Propidium iodide) and caspase-cleaved cytokeratin 18 (M30-antigen) analyses were used. In addition, apoptosis was investigated on gene and protein levels by PCR and Western Blotting. Soloxolone methyl decreased cell viability on cells in a dose and time-dependent manner and induced apoptosis markers. An increase in apoptotic proteins related to endoplasmic reticulum stress (IRE1-α, Bip, CHOP) was also determined in MDA-MB-231 cells. Moreover, an increase of apoptotic gene expressions was determined in both cells treated with Soloxolone methyl. Advance analyses should be performed to elucidate the potential of Soloxolone methyl as an anticancer agent in breast cancer treatment.

1. Introduction

Cancer leads to the appearance of 6–7 million new cases each year in developing countries and is known to be the most common cause of death after cardiovascular diseases in the world.¹ Cancer incidence is rapidly increasing in almost every country in the world. Breast cancer is the most common type of cancer among women. In 2018, number of new cancer cases will be nearly 1.806,590 in the United States (US) and 30% of this number will be the women who suffer from breast cancer.¹ In Turkey, the cancer cases have been increased every year, while the developing countries and is known to be the most common cause of death after cardiovascular diseases in the world.²

Recent studies have identified new pathways of breast cancer, biomarkers and potentially effective treatment agents.² However, the response to treatment has not yet been fully understood. Therefore, there is still an urgent need to develop new treatment options and novel drugs.

Triterpenoids, which constitute a significant part of the plant secondary metabolites, represent a group of compounds with over 100 different skeletal structures characterized by a 30 carbon skeleton (they may also be thought of as consisting of six isoprene units).³ 18βH-Glycyrrhetinic acid (18βH-GA) is an active triterpenoid metabolite, the aglycon of the glycyrrhizin, abundantly found in the root of Glycyrrhiza glabra (typically cultivated in Europe, also called European licorice), and

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G. uralensis Fisch and G. inflata Bat (used in the Chinese Pharmacopoeia). Chinese medicine practitioners recommend the liquorice root to prolong life, treat injuries and detoxify. It is also approved by the United States Food and Drug Administration (FDA) for the inclusion of various products as nutritional supplements. The antitumor effect of 18β-H-GA was thought to be due to suppression of tumor formation and induction of apoptosis. However, triterpenoids, including 18β-H-GA, have weak effects when the biological activities of their molecular targets are taken into consideration. Therefore, these compounds are used as building blocks in order to obtain more effective analogues. Soloxolone methyl (methyl 2-cyano-3,12-dioxo-18β-olean-9(11),1(2)-dien-30-oate; SM), is a new semi-synthetic derivative of 18β-H-GA (Figure 1). A previous study showed that SM inhibits different cancer cell viability in a dose-dependent manner also induces G2/M arrest and caspase dependent apoptosis in human epidermoid cancer cells KB-3-1. As a result of advanced transcriptome analysis, it was determined that ER stress plays a central role in the molecular events of SM in tumor cells. Also, it was observed that SM has both anti-inflammatory and antitumor activities in mouse models on Krebs-2 carcinoma. In this study, we investigated the cytotoxic and apoptotic effects and mechanisms of the SM on estrogen receptor (ER) positive MCF-7 and ER negative MDA-MB-231 breast cancer cells.

2. Materials and methods

2.1. Cell culture and chemicals

Human breast cancer cells MCF-7 and MDA-MB-231 were cultured in 5% and 10% respectively. FBS (Gibco) containing RPMI media (Gibco) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), l-glutamine at 37 °C in 5% CO2 containing atmosphere. The chemical synthesis of the Soloxolone methyl (SM) has been described before. The stock concentration of the SM compound (100 mM) was prepared in DMSO (the final concentration of DMSO is 0.1%). From 1 μM to 100 μM serial dilutions were prepared in complete culture medium.

2.2. MTT viability assay

The principle of this method is based on the measurement of intracellular ATP amount in cells grown in cell culture. As the ATP level measurement is based on luminescence technology, it is much more sensitive than other viability assays and there is a perfect correlation between the numbers of viable cells. For this reason, it is more sensitive and reliable than colorimetric tests (such as MTT, MTS and XTT). In other respects, colorimetric methods may cause interference. Therefore, we believe that the ATP test is very important in terms of the reliability of the test result. Our laboratory always uses these two tests together in order to validate the results.

For the ATP Analysis same culture conditions and treatment options were used as mentioned in MTT Analysis. At the end of the treatment period, the ATP assay was performed according to manufacturer’s instructions with small modifications (ATP Bioluminescent Somatic Cell Assay Kit, Sigma, St. Louis, MO, USA). Briefly, 50 μl tumor cell extraction reagent was added into each well in order to extract intracellular ATP content of the cells. Then, cell culture plates were kept at the room temperature for 20 min. After incubation, 50 μl of content from each well was transferred to a white microplate and measured using a luminometer (Bio-Tek, USA) with a luciferin-luciferase bioluminescence reaction as shown below, with a measurement time of 1 s. The results were taken as Relative Light Unit (RLU). The % viability values were calculated according to the formula given in the MTT assay.

Luciferase + ATP + Luciferin + O2 →→→→ AMP + 2Pi + CO2 + Photon (RLU)

2.3. ATP viability assay

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2.4. Fluorescence imaging for determination of cell death mode

This method is based on the detection of nuclear morphology and membrane integrity using fluorescent dyes in the cell culture conditions. Hoechst 33342 fluorescence dye can bind the DNA in both dead (apoptotic and necrotic) and alive cells. While the nuclei of necrotic cells appear larger and pale, the nuclei of apoptotic cells appear smaller and brighter than control cells. Despite that, Propidium iodide (PI) can pass through only damaged membrane, so it can dye only primary necrotic and late apoptotic (secondary necrosis) cells. In this direction, cells were seeded at a density of 5 × 10^4 cells per well into 96 well-plate and treated with 10 μM of SM for 24 and 48 h. After the treatment, cells were
incubated with Hoechst 33342 and PI dyes for 20 min at 5 μg/ml and 1 μg/ml concentrations, respectively. Then, cells were visualized with a fluorescent microscope.

2.5. M30 (caspase cleaved cytokeratin-18) method

Cytkeratin (CK) is a protein belonging to the intermediate filament family of proteins used in cancer diagnosis. CK18, an important protein of cytoskeleton during apoptosis, breaks down only by the action of caspases, an enzyme group that is activated in apoptotic cells, to form cleaved CK18 (CK18-Asp396). The M30 monoclonal antibody recognizes CK18 as an apoptotic marker, particularly by recognizing the fragment (M30 antigen) of Asp396 of CK18. Thus, cleaved CK18, an apoptosis-specific marker, is detected by ELISA. MDA-MB-231 cell line was not inappropriate for the M30 study because of to have low cytkeratin 18 expression. MCF-7 cells were treated with 10 μM dose of SM and M30-Apoptosense ELISA kit (Peviva, Sweden) was used for this method.

2.6. Flow cytometry (annexin V, caspase 3/7, mitopotential, oxidative stress)

MCF-7 and MDA-MB-231 cells were treated with 10 μM dose of SM and incubated for 24 h. Annexin V staining, caspase 3/7 activity and ROS production were studied by flow cytometry method. As a control group, untreated cells were used. At the end of the treatment period, the cells were resuspended and incubated with antibodies which are specific for the cell surface. These antibodies were labeled with fluorescent dyes. The marked cells were pumped through a capillary tube named a “flow cell” under pressure and were then laser exposed. The Muse™ Cell Analyzer (Millipore, Germany) instrument and kit compatible with were used.

2.7. Western blotting

For evaluation of apoptosis PARP, caspase-8 and caspase-3 proteins were investigated in MCF-7 and MDA-MB-231 cells. In order to analyze whether apoptosis was induced by ER stress IRE1 α, BIP and CHOP protein levels were analyzed. To sum up the procedure, after treatment of the cells with 10 μM SM for 24 h, cells were lysed to allow the intracellular proteins to be released. Total protein amount was quantified by bicinchoninic acid assay (BCA). 20 μg proteins were loaded for each sample on 10% SDS-polyacrylamide gels and then electrophoresis was executed. After the transfer of proteins, membranes were blocked and protein bands detected with mentioned antibodies. Chemiluminescence signal visualized with Fusion FX-7 (Vilber Lourmat, Torcy, France).

2.8. RNA isolation and cDNA synthesis

Cells were seeded at 2 × 10^5 cells per well and treated for 24 h with 10 μM SM. At the end of the treatment period, RNA samples were obtained by using Total RNA Purification Kit according to the manufacturer’s instructions (Jena Bioscience, Germany) and RNA quality was measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). For cDNA synthesis, 500 ng RNA was used for all treatment groups and the synthesis was performed using the SCRIPT cDNA Synthesis Kit (Jena Bioscience, Germany). For Real-time PCR analysis, LightCycler 480 Real-Time PCR (Roche, Switzerland) was used. Primers used for real-time PCR analysis have been reported in our previous study. Experiments were performed duplicated and repeated two times. For the gene expression analysis graph, SM treated groups were compared with the control group (untreated group). We count minimum 1.5-fold increase as significances.

2.9. Statistical analyses

All statistical analyses were performed by using the SPSS 23.0 statistical software for Windows. The significance was calculated using one-way analysis of variance (ANOVA). A value of p < 0.05 was considered statistically significant. Results are expressed as mean values plus/minus standard deviation and they were obtained from at least two independent experiments.

3. Results and discussion

3.1. Soloxolone methyl decreases viability of breast cancer cells

The cytotoxic effect of the SM compound was examined by MTT assay on MCF-7 and MDA-MB-231 cells and the results were validated by comparison with the results of ATP assay. After 24 and 48 h treatments, cell viability was evaluated for different concentrations (1, 10 and 100 μM) of SM compound. As seen in Figure 2, SM effectively reduces cell viability in breast cancer cells depending on the dose and time. These reductions are more clearly seen in the ATP test result (Figure 2B). As a result of 48 h SM treatment, it was determined that MCF-7 cell viability was significantly decreased at all doses (p < 0.05). Maximum inhibition of cell viability is observed at 100 μM doses. IC_{50} (50% inhibition of cell growth) values were calculated on the basis of the ATP assay results and were shown in Table 1. The IC_{50} value was of MCF-7 4.22 μM for MCF-7 cells, 5.97 μM for MDA-MB-231 cells at 48 h.

According to literature knowledge, this is the first study of the SM compound, as a 18β-glycyrrhetinic acid derivative, performed with different breast cancer cell lines. Sharma et al. (2012) reported an IC_{50} value of 18β-HGA was 32.6 μM for the MCF-7 cell line and no toxic effects on the normal mammary epithelial cells MCF-10A. Growth inhibition and apoptosis inducing effect of newly synthesized 18β-HGA derivatives on many cancer cell types were evidenced in many studies. For example, Li et al. showed that IC_{50} values of 18β-HGA derivatives, modified at C-30 with feric acid analogs and at C-3 with amino acids, were ranging from 0.49 to 29.63 μM in MCF-7 and MDA-MB-231 breast carcinoma cells. In another study, IC_{50} values on human epidermoid cancer cell line were found to be between > 10 μM and 0.3 μM for 18β-HGA and its six different derivatives, including SM. Also, it was observed that SM was effective in MCF-7 breast cancer, but the lowest IC_{50} value was found in cervical cancer cells. In a previous study, the effects of GA and its derivatives were examined. According to this study, IC_{50} values were found to be between > 10 μM and 0.3 μM for GA and its six different derivatives on human epidermoid cancer cell line. In this screening study, it was observed that SM was effective in MCF-7 breast cancer, but the lowest IC_{50} value was found in cervical cancer cells. Therefore, advanced cytotoxic analyzes have been performed in cervical cancer cells and it has been reported to induce apoptosis in these cells. The same group carried out molecular pathway analyzes of SM in cervical cancer cells in their next study and it was shown that SM induces apoptosis in cells through ER stress.

3.2. Soloxolone methyl causes apoptosis

MCF-7 and MDA-MB-231 cells were treated with 10 μM SM for 24 and 48 h as described above. At the end of the treatment period, nuclear morphologies and membrane integrity were visualized using Hoechst 33342 and PI fluorescent dyes. After treatment for 24 h, pycnotic nuclei and PI negativity, signs of early apoptosis in cells were observed (Figure 3). After treatment for 48 h, the rate of formation of pycnotic nuclei and PI negativity were increased in time depended manner indicating secondary necrosis (late apoptosis). To confirm the presence of apoptosis, M30 (caspase-cleaved cytokeratin-18) ELISA method was used. It was determined that there was no increase in M30-antigen levels after 24 h of treatment compared to untreated control, but there was a significant increase in M30-antigen.
levels in MCF-7 cells after 48 h of treatment. The measured absorbance was calculated from the formulas determined with the help of the standard curve graph and the M30-antigen levels were calculated in U/L (Figure 4). Since MDA-MB-231 cell line has low cytokeratin 18 expression, it could not be used in this study.

The only study performed about 18βH-GA or its derivatives is the investigation of the effect of 18βH-GA on the human uterine carcinoma cells of SiHa by performing Hoechst 33342 staining. According to the study, Hoechst 33342 staining in SiHa cells resulted in condensation and fragmentation, which is regarded as an apoptotic marker in the nucleus. There are no previous M30-antigen studies with SM, 18βH-GA or its derivatives.

### 3.3. Determination of apoptotic parameters

As it was indicated the apoptosis inducing effect of the SM, Flow cytometry analysis was performed to determine apoptotic parameters. Annexin V staining, caspase 3/7 activity, and ROS production were measured to confirm apoptotic cell death by Muse Cell Analyzer. MCF-7 and MDA-MB-231 breast cancer cells were treated with 10 µM SM for 24 h. Results obtained from Annexin V/7-AAD assay showed, SM compound induces apoptosis time dependent manner on MCF-7 cells. However, for MDA-MB-231 cells, while apoptosis was increased by 2.95%, late apoptosis was increased to 63.55% (Figure 5a). Besides that, caspase 3/7 activation measurement results confirmed the presence of late apoptosis. Caspase 3/7 activation was increased only in MDA-MB-231 cell line (76.4%) (Figure 5b). This may be due to the caspas-3 deficiency of MCF-7 cells. It was showed that 18αH-GA derivatives induces apoptosis through caspase activation. Haghshenas et al. studied 18βH-GA on ovarian cancer cells and they showed by Annexin V-FITC measurement that GA is the cause of apoptosis. ROS is expressed as a side-product of normal life course in cells. However, when over-production of ROS occurs; it causes considerable damage, such as DNA damage and mitochondrial disorders, leading to death of the cell. Our results suggest that ROS production is not observed in MCF-7 cells. However, 49.6% of ROS production is observed in MDA-MB-231 cells. This shows MDA-MB-231 cells are under oxidative stress. In a study with MDA-MB-231 breast cancer cells, 18βH-GA has been shown to increase ROS levels. The possible explanation of the difference between MCF-7 and MDA-MB-231 cell line can be the ER. MCF-7 is ER(+) cell line. It was shown in a study that ER-mediated mechanism can affect oxidative stress.

### 3.4. Expression profiles of death genes

The effect of SM treatment on the expression of some cell death genes (ATG3, ATG5, BECN1, TNFRSF10A, TNFRSF10B, FAS, HRK, MLKL, RIPK, PARP1) was examined using 10 µM doses of SM to determine gene expression profiles for the cells. In PCR analyses, values of 1.5 and above

![Figure 2.](image-url)
were interpreted as meaningful in gene expressions. According to the results of gene expression analysis, a fold increase of TNFRSF10B (pro-apoptotic) gene expression was observed at the end of the 24 h treatment with SM in MCF-7 cell line compared to the control. There was also a significant increase in HRK (pro-apoptotic) gene expressions in MCF-7 cells (Figure 6.A). However, as a result of 48 h of treatment of SM on both cells; it has been found that BECN1 (autophagic) and HRK (apoptotic) gene expressions in MDA-MB-231 cells and MLKL, PARP (apoptotic) and RIPK (necrotic) gene expressions in MCF-7 cells are downregulated (Figure 6.B).

MDA-MB-231 cells show an increase in BECN1 gene expression which is an autophagic gene after 24 h treatment with SM. However, after 48 h of treatment, there is a decrease in gene expression levels in the BECN1 gene for both cell line but it is more significant for MDA-MB-231 cells. There is also a decrease in MLKL, PARP and RIPK gene expression levels. Autophagy can sometimes serve as a life or death path, depending on cellular conditions. BECN1, an autophagic gene with increased expression in MDA-MB-231 cells, is responsible for the synthesis of the Beclin1 protein, a component of the PI3K (phosphatidylinositol-3-kinase) complex that controls vesicle trafficking. In this case, it was thought that the increase in BECN1 at 24 h could be a lifetime path, but when the treatment time was extended, it could not cope with the toxicity of the cell and completely removed the autophagic pathway. It was found that 18βH-GA triggered autophagy in hepatocellular carcinoma and breast cancer cells for 24 h treatment.

3.5. Soloxolone methyl stimulates ER stress-induced apoptosis

MDA-MB-231 and MCF-7 cells were applied with 10 µM SM and protein expression levels was analyzed at the end of 24 h treatment. According to the results (Figure 7), the expression level of the cleaved PARP, which is examined as an apoptosis marker in the MCF-7 and MDA-MB-231 cells, was increased compared to the untreated control. Previously Jutooru et al. showed that there was an increase in the level of cleaved PARP protein levels with GA derivatives (methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) and 3,11-dioxo-18β-olean-1,12-dien-30-oate (DODA-Me)) on pancreatic cancer cell lines. When the levels of caspase-8 and caspase-3, which are other apoptotic markers, were examined, the inactive forms pro-caspase-8 and pro-caspase-3 levels decreased in the treatment groups when compared to control cells. However, this reduction appears to be reflected only in the level of active caspase forms in the MDA-MB-231 cell line (Figure 7). Decreases in protein levels at pro-caspase levels may still be interpreted as activity gains, although they do not reflect on the active form. That is, SM is thought to induce apoptosis through the exogenous pathway as a result of 24 h treatment with 10 µM SM.

ER stress is induced by ER stress molecules IRE1-α, CHOP and GRP78/Bip are known to play roles in ER stress-induced apoptosis. Western blot results revealed that the changes in IRE1-α, BIP and CHOP protein expression levels were significant increases only in MDA-MB-231 cells (Figure 7). However, there was no significant change in ER stress markers IRE1-α, BIP and CHOP levels. In this case, suggested that apoptosis in MCF-7 cells were not associated with the ER stress mechanism.

GRP78/Bip (78 kDa glucose-regulated protein/binding immunoglobulin) is found in non-stress conditions bounded to ATF-6 (activating transcription factor 6) and IRE1-α (inositol-requiring transmembrane kinase/endoribonuclease 1o). In the stressed conditions, the separation of GRP/Bip from ATF-6 and IRE1-α initiates transcription by the passing ATF-6 through to the nucleus, resulting in IRE1-α dimerization and
autophosphorylation. Activation of the kinase domain of IRE1-α results in the activation of the JNK signaling pathway. In summary, receptors on the ER membrane, such as IRE1-α, may trigger apoptosis when cells fail to cope with stress. Taking these molecules into account, IRE1-α and GRP78/Bip are thought to go through the JNK signaling pathway via TRAF2 to pathway for apoptosis. CHOP (C/EBP homologous protein) was the first protein identified in ER-induced apoptosis pathway. CHOP level is low in cells under low ER stress conditions but when ER stress increases, CHOP level is induced by IRE1-α, PERK, and ATF-6. Increased expression of CHOP protein leads to induction of apoptosis.

Figure 5. Flow cytometry analysis with 10 µM Soloxolone Methyl (SM) on MDA-MB-231 cells for 24 h using a: Annexin V; b: Caspase 3/7; c: Oxidative stress assays.

Figure 6. Changes in gene expression profiles determined by real time PCR method after A: 24 h and B: 48 h of 10 µM Soloxolone Methyl (SM) treatment.
apoptosis in many cell lines, while apoptosis resistance is indicated in cells lacking CHOP protein.40,53 Zhu et al. GRP78/Bip, PERK, ERP72 were investigated for proteins associated with ER stress on A549 and H460 lung cell line which treated with GA, and it was determined that GA causes ER stress due to the increase in these proteins. In addition, when the ER stress mechanism is inhibited by 4-phenylbutyric acid, it has been shown that ER stress proteins and apoptosis are reduced and the efficacy of ER stress has been proved.55,56,57

4. Conclusion

In conclusion, it has been shown that SM compound, a semi-synthetic derivative of 18β-glycyrrhetinic acid obtained by direct chemical modification of the triterpenoid scaffold, has high cytotoxic activity in MCF-7 and MDA-MB-231 human breast cancer cells. In addition, it has been determined to apoptosis triggered by ER stress in MDA-MB-231 cells. Our results show that SM can be promising for cancer therapy and more experiments in vivo are needed to understand its anticancer effects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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