β -Escin Reduces Cancer Progression in TNBC by Inhibiting Glutamine Metabolism Through Downregulation of c-myc.

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Abstract

The oncogene c-myc, which causes glutamine dependence in TNBC, is also the target of one of the β -Escin affected signaling pathways. We sought to determine how c-myc protein affects glutamine metabolism and proteins ASCT2 and GLS1 in β -Escin-treated MDA-MB-231 cells by glutamine uptake and western blot analysis. We also evaluated cell viability, colony formation, migration and apoptosis in MDA-MB-231 cells in response to β -Escin treatment using MTS, colony forming, wound healing, and Annexin-V assay. We determined that β -Escin decreased glutamine uptake and decreased myc and GLS1 protein expressions, conversially increased the expression of ASCT2. In addition, we determined that this inhibition of glutamine metabolism decreases cell proliferation, colony formation and migration, and induces apoptosis. In this study, it has been revealed that β -Escin inhibits glutamine metabolism through c-myc in MDA-MB-231 cells, by causing a reduce in the carcinogenic properties of the cells as a result of interrupting the energy source of these cells. As a result, β -Escin can be used as a therapeutic agent in glutamine dependent cancers.

Introduction

Breast cancer, which is complex, multifactorial and highly heterogeneous, is one of the most common cancers worldwide. Breast cancer displays clinical heterogeneity in the expression of genes related to energy metabolism[1, 2]. Breast cancer subtypes are also quite heterogeneous in terms of intake and use of glutamine, which is the second most important energy source for some cancer types[1, 3]. Compared to other breast cancer subtypes, triple negative breast cancer (TNBC), which metastasizes to distant tissue, has very poor prognosis, is aggressive, and has exogenous glutamine dependence[3, 4]. Extracellular glutamine is taken into the cell with the Na⁺ associated glutamine transporter alanine-serine-cysteine 2 (ASCT2) protein upregulated by myc[5]. Glutamine taken into the cell is converted into glutamate by the glutaminase (GLS) enzyme, which is in the first step of metabolism[6]. There are two different forms of the GLS enzyme; kidney type glutaminase (GLS1, KGA or GAC) encoded by GLS [7] and liver type glutaminase (LGA or GAB) encoded by GLS[8]. GLS2 is regulated by p53 tumor suppressor and exhibits tumor suppressive effects[9], while GLS1 expression is upregulated in many cancer types such as breast, colon, pancreas, glioma and lung cancer[10–13]. Suppression of glutamine metabolism, which contributes to the progression of glutamine-dependent tumors, is important in reducing the aggressiveness of these tumors and determining therapeutic approaches[14].

Escin is a natural triterpene compound obtained from the seeds of *Aesculus hippocastanum*. Escin has been used in traditional medicine treatments for centuries [15] and in clinical practice in cases of venous insufficiency in hemorrhoids [16], hematoma, trauma or postoperative surgery[17, 18]. It was also shown to have anti-proliferative and apoptosis-inducing properties in many types of cancer such as renal cancers [19], leukemia[20–22], breast cancer [20], cervical cancer [20], glioma and lung cancer[23]. It was noticed that β -Escin induced apoptosis through the ATM/H2AX pathway in colorectal cancer cells [24], and through the ROS/P38 MAPK pathway in osteosarcoma cells[25]. It inhibited metastasis and angiogenesis through the NF- κ B/I κ B signaling pathway in melanoma cells[26], and had anti-proliferative

effects in hepatocellular carcinoma with STAT3 inhibition[27].Furthermore, it β -Escin decreased c-myc expression, a regulator of glutamine metabolism, one of the disrupted energy metabolisms which is a fundamental feature of cancer progression in various cancer cells[28, 29].

Therefore, the effects of β -Escin were investigated on expression of c-myc, GLS1,ASCT2 and cell proliferation, migration,apoptotic cell death and glutamine metabolism in aggressive MDA-MB-231 breast cancer cells. Our findings demonstrated that β -Escin treatment suppressed the expression of c-myc, GLS1 and ASCT2[25], suggesting reduced glutamine metabolism in TNBC cells. Overall, the results demonstrated that β -Escin treatment leads to reduced cell proliferation, migration/invasion and induced apoptotic cell death through suppression of c-myc expression and glutamine metabolism.

The results indicated that β -Escin treatment may reduce exogenous glutamine intake or glutamine metabolism, which is a potential molecular target to control growth of aggressive breast cancer cells.

Material And Methods

Cell Culture and Drug Treatment

MDA-MB-231 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured as described in previous studies [30, 31]. β -Escin was purchased from USP Reference Standard (Cat no: 1249202) and dissolved in dimethylsulfoxide (DMSO; Merck).

Detection of cell viability and proliferation

To detect the effect of β -Escin on cell proliferation of MDA-MB-231 cells, we performed MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) analysis. MTS analysis was done as described in previous studies in cells treated with different doses of β -Escin at 24, 48 and 72 hour intervals [30, 31].

β -Escin stock solution was dissolved to 1 mM in DMSO and stored at -80 °C. In the study, the DMSO solvent group was used as the control group and evaluations were made according to the control group.

Western Blot Analysis and Antibodies

MDA-MB-231 cells were treated with β -Escin at 48 h and 16–18 μ M concentrations, and western blot analysis was done to define the expression levels of the targeted proteins (c-myc, ASCT2 and GLS1). Lysis of cell samples, total protein extraction and measurement, separation of proteins in SDS-Page and transfer to the membrane, antibody treatment and imaging of target proteins in Chemiluminescence were carried out to determine target protein expressions, as stated in previous studies [32].

Glutamine uptake

To detect the effect of β -Escin on glutamine uptake of MDA-MB-231 cells, breast cancer, glutamine uptake analyses were performed. Cells were seeded at a density of 1500 cells/well in 96-well plates and β -Escin treatment was administered at the indicated times and concentrations. After 96 hours incubation, 2 μ l of

cell medium was removed and assay steps were followed according to the manufacturer's protocol (Glutamine/Glutamate-Glo™ assay; Promega). Luminescence measurement was determined using a microculture plate reader (Promega, Glomax-Multi Detection System).

Colony Forming Assay

To detect the effect of β -Escin on colony formation of MDA-MB-231 cells, clonogenic assay was performed. MDA-MB-231 cells were seeded in 6-well plates and treated with β -Escin at the detected concentrations at 48 h of culture and incubated for 14 days. After incubation, cells were washed with PBS and stained with crystal violet to count visible colonies [30].

Apoptosis Analysis

Muse Annexin V apoptosis and dead cell assay reagent were used for apoptosis analysis (Millipore; MCH100115). MDA-MB-231 cells were seeded in 25 cm² flasks (3.5×10^5 cells/4 ml medium) and treated with β -Escin concentrations for 48 h. Then cells were harvested by removing with trypsin. Afterward, the cells were stained with annexin V and dead cell reagent according to the manufacturer's protocols (Millipore Corporation) and analyzed using Muse Cell Analyzer (Millipore Corporation).

Scratch Wound Healing Assay

To detect the effect of β -Escin on migration of MDA-MB-231 cells, we performed wound-healing scratch assay. MDA-MB-231 cells were seeded in 6 well plate (3×10^5 cells/wells), and treated with β -Escin at the indicated concentrations (16–18 μ M) for 48 h. Then the cell monolayer was wounded using a 100 μ l sterile pipette tip and the cell medium was replaced with fresh medium. Wound areas forming in the cell monolayer were imaged at 0, 24 and 48 h with a phase-contrast microscope and multiple images of the wound areas were measured using Image J program [30].

Statistical Analysis

All experiments were performed with at least three repetitions. Statistical analyses were evaluated using GraphPad Prism 6. Data were expressed as mean $\pm$ SD and evaluated using the unpaired t test. $p < 0.05$ was considered statistically significant.

Results

β -Escin treatment suppresses proliferation and colony formation in MDA-MB-231 cells

To determine the effects of β -Escin on MDA-MB-231 cell proliferation and viability, MTS assay was performed after 48 h treatment with increasing doses of β -Escin (16–18 μ M). The MTS analysis revealed that β -Escin treatment significantly reduced cell proliferation of MDA-MB-231 (Fig. 1a) compared to DMSO-treated control cells. We then investigated the effects of β -Escin on colony formation of MDA-MB-

231 cells. β -Escin treatment (10–14 μ M) led to a significant reduction in the number of colonies compared to DMSO-treated cells (Control) (Fig. 1b-1c)

β -Escin treatment inhibits the migration of MDA-MB-231 cells

To determine the effect of β -Escin on cell motility and migration of MDA-MB-231 cells, wound healing analysis was performed. β -Escin treatment (16–18 μ M) significantly reduced motility or migration of MDA-MB-231 cells (Fig. 2a-b) compared to control cells.

β -Escin treatment induces apoptotic cell death in MDA-MB-231 cells

In order to determine the effect of β -Escin on apoptosis in MDA-MB-231 cells, concentrations of β -Escin from 16–18 μ M, which were determined to reduce cell viability by 50% in MDA-MB-231 cells, were used for treatment for 48 h. Control and β -Escin-treated cells were measured with annexin V staining in flow cytometry. The rate of apoptosis was significantly increased in β -Escin-treated cells compared to controls (Fig. 4a-b). As a result, the treatment of β -Escin on MDA-MB-231 cells induced apoptosis in these cells.

β -Escin treatment inhibits expression of c-myc and molecules associated with glutamine metabolism and glutamine metabolism in MDA-MB-231 cells

In cancer cells, c-myc plays a role as an important regulator of energy metabolism. It specifically induces mitochondrial biogenesis and regulates GLS1 protein levels via miR-23a/b [28]. Therefore, we analyzed the expression of c-myc in β -Escin-treated MDA-MB-231 cells using western blot. As shown in Fig. 3a-b, β -Escin treatment led to decreased expression of c-myc. Furthermore, we investigated expressions of GLS1 and ASCT2 proteins, which are associated with glutamine metabolism, in β -Escin-treated cells. We found that β -Escin treatment also inhibited expression of GLS1 (Fig. 3a-c), but ASCT2 expression increased compared to controls (Fig. 3d).

Next, glutamine uptake analysis was performed to determine if glutamine uptake was altered by inhibition of c-myc expression in β -Escin-treated cells. The amount of glutamine in the culture medium of β -Escin-treated cells and control cells was determined with the relative light unit (RLU) values obtained as a result of luminescence measurements. The RLU values showed that the amount of glutamine in culture medium of the β -Escin-treated cells was higher than the controls (Fig. 3e). Our results indicate that the glutamine uptake decreased in β -Escin-treated cells compared to control cells.

Discussion

Glutamine dependence is due to mutations that result in activation of the c-myc oncogene or loss of tumor suppressors such as Rb, which triggers glutamine uptake and utilization through up-regulation of enzymes and cellular transporters that support replication in tumors, causing an aggressive cancer phenotype [33, 34]. Breast cancer, with a heterogeneous tumor character, is also heterogeneous in terms of glutamine metabolism. The c-myc oncogene, which is associated with reprogramming in tumorigenesis, is overexpressed in 40% of TNBCs, making these cells glutamine dependent [35]. The glutamine-dependent

phenotype of TNBC tumors provides an opportunity to offer effective therapies targeting c-myc and glutamine metabolism-related enzymes as the main susceptibility of these tumors. In this respect, β -Escin has anti-proliferative properties through the pathways it affects in many cancers.

Escin induced DNA damage and apoptosis via the ATM/H2AX pathway at large amounts in HCT116 colorectal cancer [24]. Wang et al [25] stated that Escin Ia suppressed the metastasis of triple negative breast cancer cells by inhibiting epithelial mesenchymal transformation [36]. In in vivo and in vitro studies with human osteosarcoma cells, autophagy and caspase-dependent apoptosis were induced via the ROS/P38 MAPK pathway. In B16F10 and SK-MEL5 melanoma cells, β -Escin was suggested to inhibit anti-tumorigenic properties such as metastasis and angiogenesis via NF- κ B/I κ B signaling [37]. Wang et al [26] showed that β -Escin inhibited NF- κ B and NF- κ B-regulated gene products such as c-myc, Cyclin D1, COX2, Survivin, and Bcl-2 in pancreatic cancer. β -Escin applied to hepatocellular carcinoma cells inhibits signal transducer and activator of transcription 3 (STAT3) and downregulates the expression of STAT3-regulated gene products; thus, suppressing cellular proliferation and providing chemotherapy sensitivity [27]. Huang et al [38] stated that β -Escin administration to cholangiocarcinoma cells reversed P-gp-dependent multidrug resistance by inhibition of the GSK3 β / β -catenin pathway, and they stated that the use of β -Escin in combination for the treatment of these cancers is promising. Considering the signaling pathways in which β -Escin acts on cancer cells, we determined that β -Escin suppresses glutamine metabolism via c-myc in MDA-MB-231 cells; thus, reducing the proliferation, clonogenicity and migration of these cancer cells.

Glutamine metabolism-related proteins show heterogeneity in breast cancer subtypes. van Gedelsman et al [39] stated that ASCT2 has high expression in most breast cancers, but ASCT2-mediated glutamine uptake is required for maintenance of cancer progression and mTORC1 signaling only in TNBC. In this study, which we performed with MDA-MB-231 cells, it is understood that the amount of glutamine taken into the cells decreased in the cells treated with β -Escin, but this situation is not related to the protein levels of ASCT2. It was thought that the only reason for the reduction in the amount of glutamine uptake into cells without reduction in ASCT2 protein levels was the GLS1 levels in β -escin-treated cells.

In this study, it was determined that β -Escin regulates the expression of c-myc and c-myc in MDA-MB-231 cells showing glutamine dependence, and protein expression of the GLS1 enzyme decreased. Glutamine utilization is also reduced in MDA-MB-231 cells in connection with the decrease in c-myc and GLS1 expression, but this decrease in glutamine utilization is not related to the ASCT2 protein in the cell membrane. Similar to other studies showing the anti-proliferative effects of β -Escin, we determined that treatment of MDA-MB-231 cells exhibited cytotoxic properties and inhibited colony formation and migration. We also showed that it induces apoptosis.

In this study, β -Escin inhibited the glutamine pathway, which meets the survival and proliferation needs of these cells, by reducing the use of glutamine through suppression of myc in glutamine-dependent MDA-MB-231 cells. In this way, β -Escin suppressed glutamine metabolism in MDA-MB-231 cells and reduced carcinogenic properties. β -Escin offers a glutamine anti-metabolic feature that can be used in cancer

treatment, such as for TNBC which is glutamine dependent, by directly or indirectly triggering the c-myc oncogene due to the pathways it affects.

Declarations

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Conflict of interest:

The authors have no conflicts of interest or other disclosures to report.

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Not applicable

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All authors contributed equally to this work.

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This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

Figure 1

β -Escin inhibits cell proliferation and colony formation of MDA-MB-231 cells. a) MDA-MB-231 cells were seeded at a density of 1.5×10^3 cells/well into 96-well plates and treated with a series of concentrations of β -Escin (16,17, and 18 μ M) or a vehicle only control for 48 h and cell viability was measured by MTS assay. b) A total of 1000 cells were seeded in six-well plates and cultured for 10-14 days. Colony formation was detected. c) Quantitative analysis of the colony numbers of MDA-MB-231 cells after β -Escin treatment. The values are mean $\pm$ SD from three independent experiments. ***p<0.001, **p<0.01 vs the control group.

Figure 2

β -Escin inhibits cell motility and migration in MDA-MB-231 cells. Cell migration in cells treated with β -Escin at concentrations of 16, 17, and 18 μM was determined by scratch wound healing analysis. a) Cells treated with β -Escin for 48 h were wounded with a 100 μl pipette tip. Microscopic images were taken at 0, 24 and 48 h to determine the cell migration in the created wound areas. b) Quantitative analyses of wound areas in Escin-treated cells were compared with the control group treated with DMSO. *** $p < 0.001$, ns; not significant.

Figure 3

β-Escin suppresses glutamine metabolism by downregulating c-myc protein expression. MDA-MB-231 cells were treated with 16, 17, and 18 μM concentrations of β-Escin for 48 h. a) c-myc and glutamine metabolism-associated GLS1 and ASCT2 protein expression in MDA-MB-231 cells treated with the concentrations of 16, 17 and 18 μM β-Escin was determined by western blot analysis. b) Quantitative analysis of the optical density ratio of c-myc compared with the loading control (tubulin) in MDA-MB-231 cells. c) Quantitative analysis of the optical density ratio of GLS1 compared with the loading control (tubulin) in MDA-MB-231 cells. d) Quantitative analysis of the optical density ratio of ASCT2 compared with the loading control (tubulin) in MDA-MB-231 cells. e) Glutamine uptake in MDA-MB-231 cells after β-Escin treatment was determined by measuring the relative light unit (RLU) by luminescent imaging. The values are mean ± SD from three independent experiments. ***p < 0.001 vs the control group.

Figure 4

β-Escin induces apoptosis in MDA-MB-231 cells. Cell death by apoptosis in cells treated with β-Escin at concentrations of 16, 17, and 18 μM for 48 h was determined by Muse Cell Analyzer using Annexin V/PI staining. a) Histogram of apoptotic cells in β-Escin-treated cells after measurement. b) Analysis of apoptotic cell numbers in β-Escin-treated cells compared to control group. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$