

Investigation of the Anticancer Effect of S-Allyl-L Cysteine on the C6 Rat Glioma Cell Line *in vitro* in 2D- and 3D-Cell Culture Models

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ABSTRACT

AIM: To investigate the antiproliferative and apoptotic effects of S-allyl cysteine (SAC) on C6 glioblastoma cells using two- and three-dimensional (2D and 3D) cell culture systems.

MATERIAL and METHODS: Three groups of rat glioma cell line C6 were prepared: 2D-Control, 2D-SAC, 3D-CMC-Control, and 3D-CMC-SAC. The control cells were incubated under standard culture conditions, the SAC cells were incubated in a culture medium supplemented with the IC50 dose (50 µM for both the 2D-SAC C6 and 3D-CMC-SAC groups) of SAC for 24 and 48 h. All experimental cells were stained with antibodies recognizing NOTCH1 and JAGGED1, and the mRNA expression levels of NOTCH1 and JAGGED1 were evaluated by qRT-PCR.

RESULTS: Increasing doses of SAC were administered for 24 h to the C6 glioma cell line. The concentration of 50 µM was selected as the most suitable dose for administration. The gene expression profiles differed between these two cell culture types. We found that the expression levels of NOTCH1 receptor mRNA were lower in cells exposed to 50-µM SAC for 24 h than those of control cells in both 2D and 3D cell cultures. The immunoreactivities of both the biomarkers JAGGED1 and NOTCH1 in the glioma cells decreased significantly in the SAC group.

CONCLUSION: These findings indicate that SAC is a potential drug candidate for human use, as indicated by its nontoxic nature. In addition, SAC was found to exert an anticancer effect, which is associated with the modulation of JAGGED1 and NOTCH1 signaling pathways in glioma cancer cells.

KEYWORDS: 3D cell culture, JAGGED1, NOTCH1, S-Allyl-L cysteine

INTRODUCTION

Malignant glioma is a common type of brain tumor that is associated with a high mortality rate (3). Despite the advances in therapeutic methods, glioblastoma multiforme (GBM) remains the most malignant glioma in the past decade, with a mean survival of <24 months (2). Two Food and Drug Administration approved drugs, carmustine

and temozolomide, are currently being used to treat malignant gliomas. The glioma research is focused on enhancing the treatment efficacy.

Presently, complementary and alternative therapy (CAT) applications are extensively being used in the treatment of cancer (44). In fact, CAT is used in >50% of the patients with cancer in Europe (29). Both *in vivo* and *in vitro* studies have

exhibited the antioxidant activity of S-allyl cysteine (SAC) (10). In the literature, the anticancer, antihepatotoxic, and neuroprotective activities of SAC have been documented (5, 17).

Two-dimensional (2D) cell culture systems are frequently used to evaluate the sensitivity of tumor cells to radiotherapy and chemotherapy (18). Because these cultures do not interact with the surrounding cells or their microenvironment, they are not deemed suitable to investigate solid tumors. Drug sensitivity may vary between the *in vitro* and *in vivo* clinical evaluations (6,12). Hence, it is necessary to establish a new research model for developing an effective antiglioma therapy (18). Three-dimensional (3D) cell culture systems that can better mimic natural tumor microenvironments are used in various cancer studies. These cell culture systems have been applied for several tumor types as they better simulate the natural tumor microenvironment and facilitate more precise drug efficacy analysis (19,25,36). Biomaterials commonly used in glioma studies include matrigel and hydrogels. Their applications mainly focus on determining the sensitivity of cocultured tumor cells to radiation and drugs (19,40).

In the literature, the anticancer effects of SAC on glioma cells have generally been studied in 2D culture models. Evaluating the antitumoral effect of SAC in 3D cell models that are closer to *in vivo* models will better facilitate the transition to clinical application. In our study, we investigated the effects of SAC on C6 glioblastoma cells and the antiproliferative and apoptotic effects of SAC in 2D- and 3D cell culture systems.

■ MATERIAL and METHODS

2D Cell Culture Conditions

The C6 glioma cell line was obtained from ATCC (Manassas, VA, USA). The cells were prepared to proliferate in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics. The cultures were grown at 37°C in a humid incubator with a 5% CO₂ atmosphere and 95% humidity. The cells were subcultured at 48-h intervals with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and cultivated in fresh media. The cells were regularly evaluated for mycoplasma contamination.

Hydrogel Production

Carboxymethyl cellulose (CMC) hydrogel was prepared in 3 different proportions: 1%, 2%, and 3% w/v. Distilled water was heated to 70°C, and then, CMC powder was added to the solvent and mixed for 30–60 min. After the distilled water was heated to >70°C in a beaker, the CMC powder was added to the solvent and mixed for 30–60 min to obtain a homogeneous mixture. Hydrogels prepared at different concentrations were cooled at 4°C for 24 h before use. CMC hydrogels were sterilized by autoclaving at 120°C for 15 min. The autoclaved CMC hydrogels were stored at 2°C–8°C until they were homogeneous.

Determination of the Capacity for Water Absorption of Hydrogel

To evaluate the water-holding capacity of hydrogels prepared at different concentrations (1%, 2%, and 3% (w/v)), they were prepared as circles on an equal surface area ($r=0.8$ cm) and then weighed (W_0). After the samples were kept in 2 mL of phosphate-buffered saline (PBS) for 24 h, they were removed from the solution and weighed again (W_1), and the percentage water-holding capacity was calculated using the below equation:

$$\% \text{ Capacity of Water Absorption} = \frac{W_1 - W_0}{W_1} \times 100$$

3D Cell Culture Conditions

CMC hydrogel (1% w/v) was mixed with DMEM in the ratio of 1:1 and then centrifuged at 4500 rpm for 15 min. For MTT analyses, 13 μ L of MC + DMEM hydrogel was added to each well of a 96-well plate in a bioprinter (Dual X, Axolotl Biosystems Ltd., İstanbul, Turkey) (Figure 1).

Cell Survival

After separating from the vials containing 0.25% trypsin + EDTA, the resulting cells were centrifuged for 5 min at 1200 rpm/4°C. The cells were counted with the CEDEX (Roche; Mannheim, Germany) cell counter and cultivated in 96-well plates (approximately 10^4 cells/0.25 mL). SAC (Tokyo Chemical Industry, Product Number: A1468, CAS RN: 21593-77-1, Lot. WWHCI-NQ, purity: >98.0% (T), Tokyo, Japan) was dissolved in the culture medium. The C6 cells were treated with 2.5, 5, 10, 25, 50, and 100 μ M of SAC for 24 and 48 h, and the cell viability was determined using the 3-(4,5-D-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT) test at 550 nm with a microplate reader (BioTek; Winooski, VT) *in vitro*. A minimum of 3 independent assays were run for statistical analyses.

Quantitative Reverse Transcription PCR

RNA was isolated from the cells (GeneJet RNA Purification

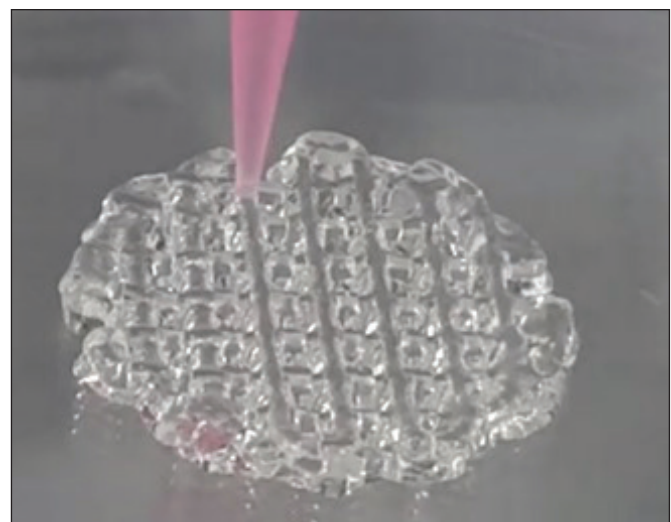


Figure 1: CMC hydrogel in a bioprinter.

Kit, Thermo Scientific, USA), and its concentration was measured (NanoDrop 1000, Thermo Scientific). The isolated RNA samples were converted to cDNA by treatment at 42°C for 60 min, followed by treatment at 70°C for 5 min as specified by the manufacturer's instruction (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). The cDNA samples were stored at -80°C. The gene expression levels of NOTCH1 and JAGGED1 were determined using the SYBR Green qPCR Kit (Thermo Scientific). cDNA synthesis was verified via detection of the β -actin transcript. The following primers were used in the real-time PCR: NOTCH1 mRNA was amplified with 5' CACCCATGAC-CACTACCCAGTT3' and 5' CCTCGGACCAATCA-GAGATGTT3' primers, and JAGGED1 mRNA was amplified with 5' AACTGGTAC-CGGTGCGAA3' and 5' TGATGCAAGATCTCCCT-GAAAC3' primers. The relative differences in the expression levels were determined by the $2^{-\Delta\Delta C_t}$ method (26).

Immunocytochemistry Staining

The experimental cells were assigned to 2 main groups: the control groups (2D-Control and CMC-3D-Control) and the SAC-treated groups (SAC and CMC-SAC). The cells in the control groups were cultured under standard conditions; the SAC-treated cells were incubated with SAC for 24 h at the calculated IC50 values. After incubation, the cells belonging to all experimental groups were fixed with 4% paraformaldehyde for 30 min, washed with PBS twice for 30 min each, and then placed on an ice bath for 15 min with 0.1% Triton X-100 (Applichem, 4L003808) for permeabilization. The samples washed with PBS thrice for 5 min each were further incubated with 3% H₂O₂ (Merck, USA) at room temperature for 10 min to inhibit the endogenous peroxidase activity. After washing again with PBS thrice for 5 min each, the cells were incubated with blocking solution (Invitrogen, 859043) for 1 h at room temperature. Next, the blocking solution was withdrawn without washing, and the primary antibodies against NOTCH1 (C-10): sc-373891, Santa Cruz Biotechnology, Inc., Texas, USA),

JAGGED1 (1:500 floor. no. AP0531) and caspase-3 (Bioss, bs-0081R) were applied in 1:100 dilution to the cells and incubated at 4°C (16 ± 18 h). The next day, antibodies were removed by washing thrice with PBS for 5 min, followed by treatment with a biotinylated secondary antibody for 30 min. After washing thrice with PBS for 5 min each, the mixture was incubated with streptavidin horseradish peroxidase (Invitrogen, 859043) for 30 min at room temperature. After washing again with PBS, the samples that had been incubated with diaminobenzidine (DAB) (Scytek, AEM080) for 5 min were washed with distilled water (thrice for 5 min each time) to determine the visibility of the immunocytochemical reaction. The samples were stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for 2–3 min and washed for 10 min in distilled water and then covered with a covering medium (Spring Bioscience-DMM125). The results of the immunohistochemical staining were quantified using Fiji (ImageJ version 2.1.0, National Institutes of Health, Bethesda, MD, USA), as described by Patera et al. After color deconvolution was applied to the microphotographs belonging to the groups, DAB was used

to calculate the average gray values from the images. The optical density was calculated using the following formula: $\log(\text{maximum gray density}/\text{average gray density})$.

Statistical Analysis

Statistical analysis was performed using the GraphPad software. The data were analyzed using the Kruskal–Wallis test or the post-hoc Tukey or Dunn test. $P < 0.05$ was considered to indicate statistical significance. The results were presented as the mean and median ± standard error data of mean and median.

RESULTS

Capacity for Water Absorption of CMC Hydrogel

The water-holding capacities of the CMC hydrogel prepared in different proportions by weight (1%, 2%, and 3% (w/v)) were found to be 339.4 ± 1.24 , 242.5 ± 2.53 , and 151.6 ± 3.08 , respectively.

MTT Analyses

MTT analysis was performed to examine the effects on cell viability. Increasing doses of SAC were administered for 24 h in the C6 glioma cell line. Increasing doses of SAC decreased cell viability in both the 2D and 3D cell cultures in a direct proportion. The concentration of 50 μM was selected as the most suitable dose for administration (Figure 2).

Differences in mRNA Expression of NOTCH1 and JAGGED between 2D and 3D Cultures

The gene expression profiles differed between these two cell culture types. The NOTCH1 receptor mRNA levels were significantly lower in the cells treated with 50 μM of SAC for 24 h when compared with the control cells in both the 2D and 3D cell cultures, with a greater statistically significant difference in the 2D cell culture. No difference was detected between

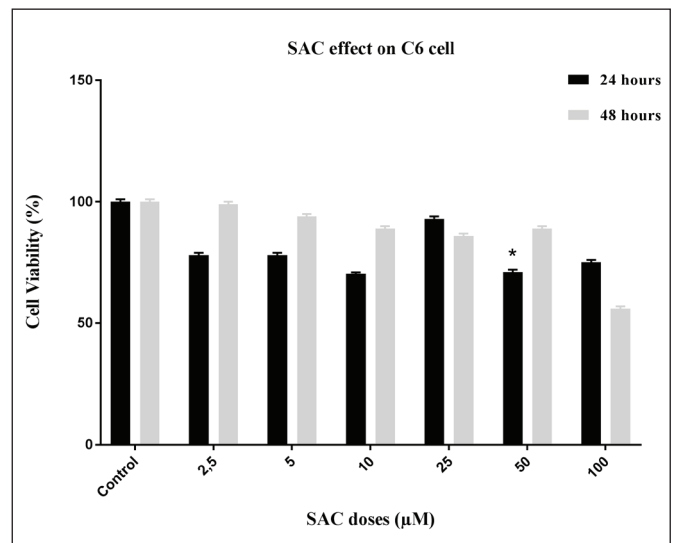


Figure 2: Cytotoxic effect of S-allyl-L cysteine (SAC) on the C6 cell line.

the 2D and 3D control groups (CMC) in terms of the NOTCH1 mRNA levels (Table I).

The expression of *NOTCH1* was evaluated and compared between the 2D and 3D culture systems in C6 cells (Figure 3).

The *JAGGED1* ligand mRNA level was significantly decreased in cells treated with 50- μ M SAC for 24 h in the 2D cell culture when compared to that of the control cells. However, no statistically significant change in the *JAGGED1* mRNA levels was noted after SAC treatment in the 3D cell culture system (Table II).

The expression of *JAGGED1* was evaluated and compared between the 2D and 3D culture systems in C6 cells (Figure 4).

Immunocytochemistry Analyses

Indirect immunocytochemical staining was performed to evaluate the effectiveness of SAC on NOTCH1 and JAGGED1 in the C6 glioma cell line. As shown in Figures 5 and 6, the immunoreactivities of both the markers JAGGED1 and NOTCH1 were significantly decreased in the glioma cells of the SAC group than that in the control group ($p < 0.05$). The qRT-PCR results were consistent with the immunocytochemical findings (Figure 7).

DISCUSSION

GBM is cancer that causes loss of neurological functions and, eventually, death as a result of infiltration of neoplastic cells into the central nervous system (14). C6 glioma cells are

employed in GBM research as cells with various malignant glioblastoma characteristics, such as high mitotic activity, nuclear pleomorphism, tumor necrosis foci, and intratumor bleeding (14).

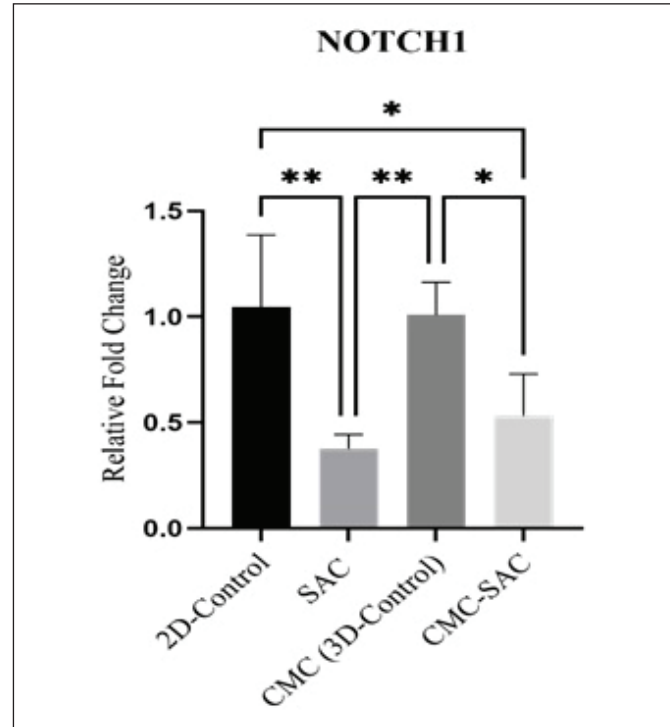


Figure 3: The mRNA expression of the NOTCH1 receptor in 2D and 3D cell cultures.

Table I: Comparison of NOTCH1 Gene Expression Levels in the 2D and 3D Cell Culture Systems

NOTCH1	p
2D-Control vs. SAC	<0.0001
2D-Control vs. CMC (3D-Control)	0.4107
2D-Control vs. CMC-SAC	<0.0001
SAC vs. CMC (3D-Control)	0.0001
SAC vs. CMC-SAC	0.9629
CMC (3D-Control) vs. CMC-SAC	0.0003

Table II: Comparison of JAGGED1 Gene Expression in the 2D and 3D Cell Culture Systems

JAGGED1	p
2D-Control vs. SAC	<0.0001
2D-Control vs. CMC (3D-Control)	0.0064
2D-Control vs. CMC-SAC	<0.0001
SAC vs. CMC (3D-Control)	<0.0001
SAC vs. CMC-SAC	0.6408
CMC (3D-Control) vs. CMC-SAC	<0.0001

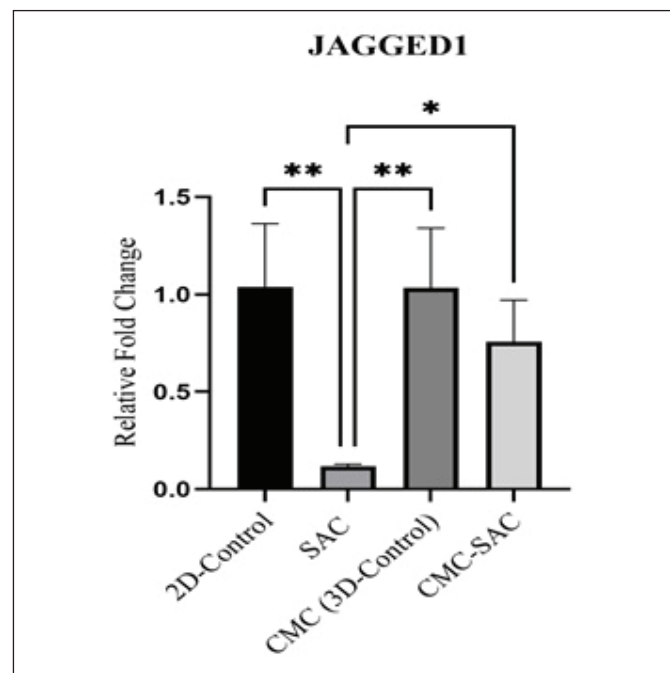


Figure 4: The mRNA expression of the JAGGED1 ligand in 2D and 3D cell cultures.

In this study, we investigated the anticarcinogenic properties of the active ingredient of SAC in C6 glioma cells cultured in 2D and CMC tissue scaffolds cultured in 3D. For this purpose, we evaluated the expression of JAGGED1 and NOTCH1 by qRT-PCR and immunohistochemical assays after treating with SAC.

The anticarcinogenic effect of aloe emodin, a plant-derived anthraquinone derivative, was investigated in C6 cells; of the doses, 20 and 40 μM of aloe emodin applied to C6 cells, the

dose of 20 μM was found to be effective (7). In another study, the effects of specific doses of vitamin K-3 on the cell survival rate of C6 cell lines were studied to determine that the dose acting on the cells was 40 μM (31). In a study conducted on bladder tumor cells, SAC was administered to tumor cells at doses of 0, 10, 25, and 50 μM , followed by quantitative measurements and cytometric analyses (15). The results were found to be statistically significant. Moreover, it has been found that SAC is anticarcinogenic, especially owing to its apoptosis-inducing

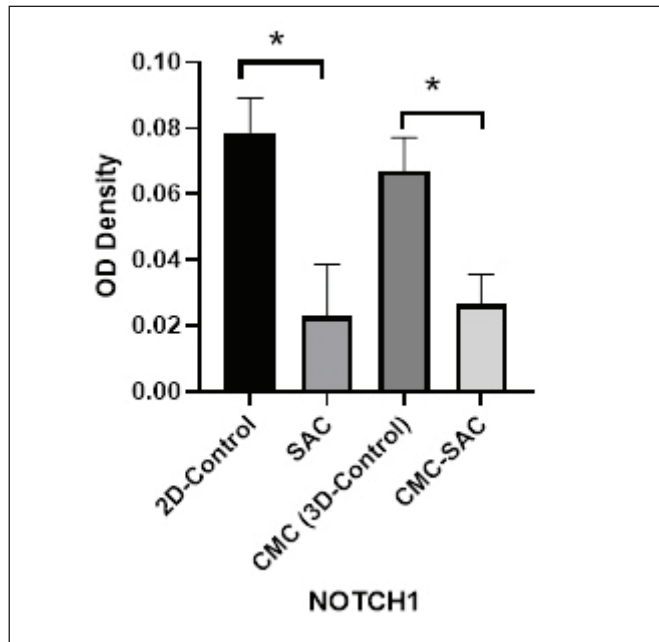


Figure 5: OD values for NOTCH1.

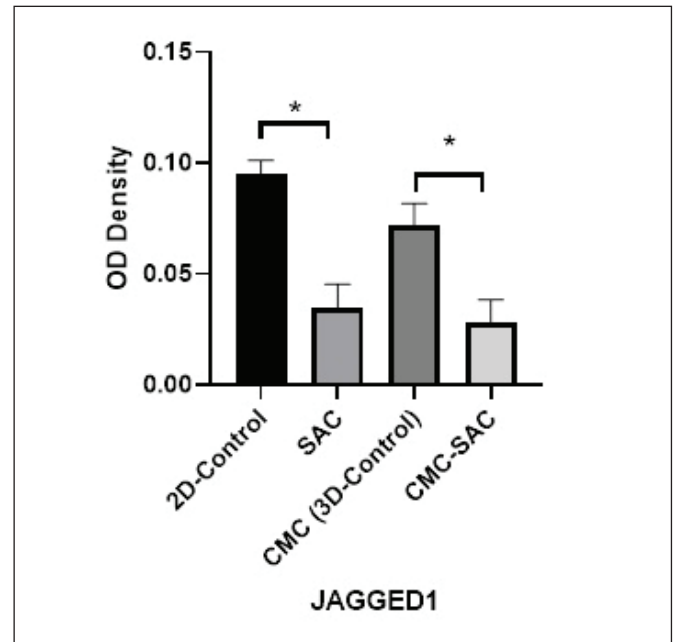


Figure 6: OD values for JAGGED1.

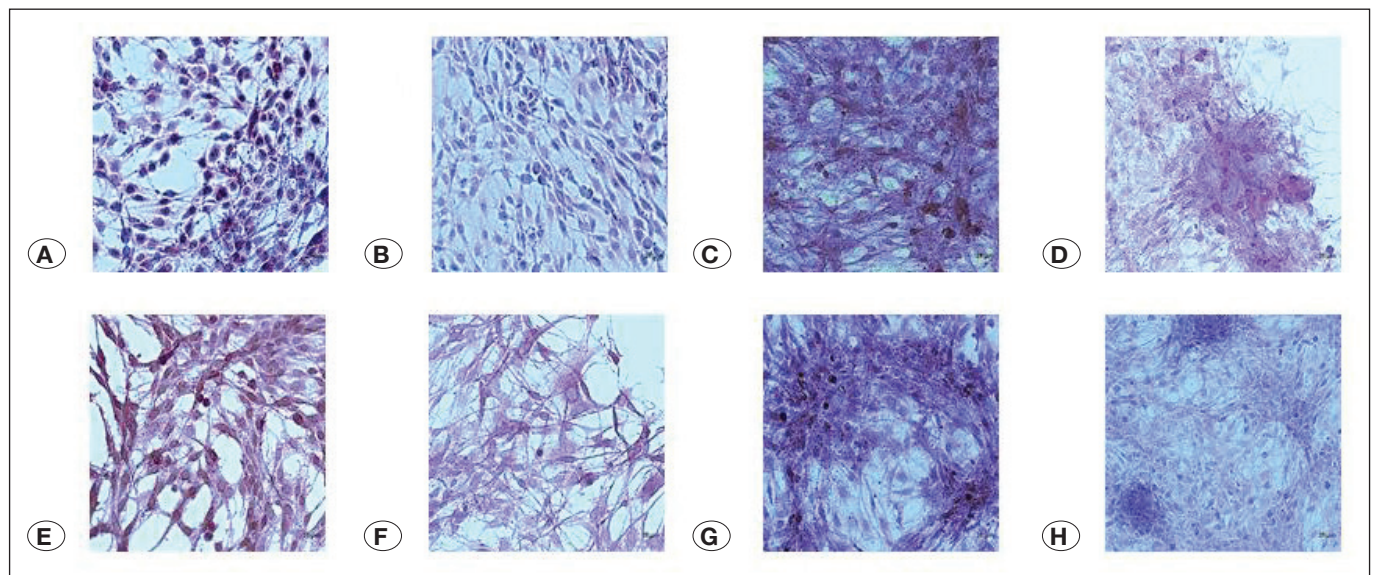


Figure 7: Immunocytochemistry staining images of the NOTCH1 receptor and the JAGGED1 ligand in 2D and 3D cell cultures. **A)** NOTCH1 expression in the 2D Control; **B)** NOTCH1 expression in the SAC group; **C)** NOTCH1 in the CMC 3D Control; **D)** NOTCH1 in the CMC-SAC group; **E)** JAGGED1 in the 2D Control; **F)** JAGGED1 in the SAC group; **G)** JAGGED1 in the CMC 3D Control; **H)** JAGGED1 in the CMC-SAC group.

effect on these cells. SAC was found to suppress proliferation and induce apoptosis in a study conducted *in vitro* in ovarian cancer cells at doses of 1–100 μ M (1). No study has yet been conducted on the anticarcinogenic effect of SAC on C6 cells. Thus, our findings on this subject will be the first contribution to the literature.

Kanamori et al. revealed that SAC induced cytotoxicity in tumor cells through the induction of the mitochondrial permeability transition (21). Welch et al. reported that SAC suppressed the proliferation of human neuroblastoma cells (41). Chu et al. demonstrated that SAC suppresses invasive growth in prostate, nasopharyngeal, and esophageal cancer cells (9). In another study, the effects of SAC on cancer proliferation and metastasis in a hepatocellular carcinoma MHCC97 cell model were investigated. The results indicated a decrease in apoptosis induction and cell proliferation (30). It has also been shown that SAC suppresses proliferation and induces apoptosis in human ovarian cancer cells *in vitro* (43).

Animal models and 2D culture systems have been demonstrated to exhibit poor similarity in the clinical outcomes (16,32,38). For testing novel antibodies, the 3D culture systems have been used owing to their similarity with the tumor microenvironments. The literature reports viability differences between the 2D and 3D cultured cancer cells (4,33). Cancer cells in the 3D models presented with greater drug resistance than those in the 2D culture (37). This difference can be explained by the limitations on mass transfer of the drugs in 3D culture systems when compared to that in 2D cultured systems (28,34).

The feasibility of using 3D models to accurately reflect tumor models has been demonstrated in studies conducting 3D coculture of cancer cells to verify the specificity of drugs in cancer cells (8,24).

Cancer cells in the 3D models are known to show phenotypic heterogeneity (22). This heterogeneity is an important factor because even in cells belonging to the same tumor group, morphological and functional changes vary with the gene expression, differentiation, and proliferation rates. Consequently, 3D models serve as suitable tools to investigate differences in the tumor microenvironment regarding gene expression (32). Moreover, it is feasible to use 3D models to accurately represent tumor models. Past studies with 3D coculture of cancer cells have been used to verify the specificity of drugs in cancer cells (8, 24).

One of the important intracellular pathways that play a role in malignant tumor pathologies is the NOTCH1 signaling pathway (27). NOTCH1 plays an important role in regulating the balance among cell proliferation, differentiation, and apoptosis (39). Previous studies have shown that cancer can develop with the disruption of NOTCH1 signaling (11).

Cell culture studies have demonstrated a link between NOTCH1 and diseases by revealing the genes and mechanisms that regulate the NOTCH1 signaling pathway (11). It has been reported that mutations in receptors and ligands

can lead to developmental disorders and the development of tumors in various tissues owing to the misregulation of the pathway (27). The major components of the NOTCH1 signaling pathway have been demonstrated to be important drug targets for anticancer therapy (23). This pathway plays a role in tumor angiogenesis and is a factor representing poor prognosis and survival. NOTCH1 ligands and receptors are transmembrane proteins (13). In a study conducted by Rodilla et al., tumor growth in mice was inhibited by the inactivation of NOTCH1 signaling as a result of the deletion of JAGGED1, a NOTCH1 ligand (35).

JAGGED1 ligands are active in GBM and low-grade astrocytoma cells (20,45). Previous studies have demonstrated the anticarcinogenic effects that can occur in pancreatic cancer cells through the regulation of the NOTCH1 and JAGGED1 pathways (42).

No study in the literature compares SAC and C6 cells. The anticancer effect of SAC has not been previously investigated using 3D tumor models. In addition, no study has yet investigated the antitumoral effects of SAC on NOTCH1 receptors and the JAGGED1 ligands. Thus, this study is the first one in the literature in this respect.

In our study, the *NOTCH1* and *JAGGED1* expression levels decreased in the group administered with SAC, demonstrating that SAC can exert therapeutic effects on tumor development by inhibiting the NOTCH1 expression in glioma cells.

This study, which explores the relationship between SAC and glioma, is important, as it is the first 3D culture study and can provide more comprehensive results. Meanwhile, the present findings are important to highlight how SAC can offer new options for cancer treatment.

■ CONCLUSION

The present results support that SAC is a potential drug for human use, as revealed by its nontoxic nature. Molecular studies have clarified the correct binding mode of SAC with NOTCH1 and JAGGED1. This binding was confirmed via *in vitro* experiments evaluating cytotoxicity and the gene expression patterns. The findings from this study validate that SAC exerts anticancer activity in glioma cancer cells by regulating the NOTCH1 and JAGGED1 signaling.

AUTHORSHIP CONTRIBUTION

Study conception and design: IK, IDC, SKS

Data collection: SKS, RO

Analysis and interpretation of results: RO, MCS

Draft manuscript preparation: IK, IDC, SKS

Critical revision of the article: IK, IDC, SKS, MCS

Other (study supervision, fundings, materials, etc...): SKS, RO, MCS

All authors (IK, IDC, SKS, RO, MCS) reviewed the results and approved the final version of the manuscript.

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