Research Square

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8β-glycyrrhetinic acid as major bioactive component of Glycyrrhiza glabra downregulates expression of CD44 and epithelial-mesenchymal transition markers and inhibits xenograft tumor growth in gastric cancer stem cells

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Abstract

Background:

Gastric cancer (GC) is a common malignancy with high rates of recurrence, metastasis, and drug resistance, often attributed to gastric cancer stem cells (CSCs). CD44, a surface marker upregulated in CSCs, plays a critical role in promoting epithelial-mesenchymal transition (EMT). 18β-glycyrrhetinic acid (GA), a major bioactive compound of Glycyrrhiza glabra, has known anti-inflammatory and anticancer properties. This study aimed to investigate the effects of GA on CD44 expression, EMT markers, and tumor growth in gastric CSCs.

Methods and Results:

Gastric CSCs were cultured in serum-free medium with EGF and B-27 under nonadherent conditions and treated with varying GA concentrations (20–160 μM) for 24, 48, and 72 hours. Cell viability was assessed using the MTT assay. mRNA expression levels of CD44, Bcl-2, and EMT markers (ZEB-1 and Snail-1) were measured by real-time PCR, while CD44 protein expression was analyzed by flow cytometry. GA's effect on tumor growth was evaluated in a xenograft mouse model. Statistical analyses included one-way ANOVA and paired t-tests, with $p \leq 0.05$ considered significant. GA reduced gastric CSC viability in a time- and dose-dependent manner. Treatment with 60 μM GA significantly downregulated the mRNA expression of CD44, Bcl-2, ZEB-1, and Snail-1, and reduced surface CD44 protein expression at 48 and 72 hours. In vivo, GA treatment inhibited tumor growth and significantly reduced tumor volume in mice.

Conclusions:

GA inhibits gastric CSC proliferation and tumor growth, potentially by suppressing EMT-related pathways via downregulation of CD44. These findings support further investigation of GA as a candidate for CSC-targeted gastric cancer therapies.

Introduction

Gastric cancer (GC) is the fifth most common and lethal malignancy worldwide and, according to GLOBOCAN 2020, the fourth leading cause of cancer-related deaths [1]. Although surgical techniques and chemotherapy for gastric cancer have advanced, chemoresistance still leads to metastasis and relapse after therapy [2]. Therefore, novel therapeutic strategies for gastric cancer are urgently needed. Recent studies have shown that resistance to chemotherapy, invasion, and metastasis are mainly due to cancer stem cell (CSC) subpopulations within heterogeneous tumors [3–6].

CSCs can be identified by specific surface markers, including CD133, CD44, CD44/CD54, CD24/CD44, and CD166 [7]. Among these, the CD44 molecule—a transmembrane glycoprotein—is the most widely

used marker for isolating CSCs from solid tumors [8]. CD44 + CSCs possess high proliferative, invasive, and migratory abilities, along with resistance to chemotherapy [9]. In fact, CD44 expression induces epithelial-mesenchymal transition (EMT), contributing to the generation, maintenance, migration, and invasion of CSCs [10, 11]. Multiple signaling pathways are involved in maintaining CSCs; for instance, aberrant activation of STAT3 prevents apoptosis by upregulating anti-apoptotic Bcl-2 family genes such as BCL-xL, BCL-2, and MCL1 [12].

One component of Glycyrrhiza glabra L. (licorice) is glycyrrhizic acid, which is metabolized into the pentacyclic triterpenoid 18β-glycyrrhetinic acid (GA). GA exhibits numerous physiological and pharmacological activities, including antimicrobial [13, 14], anti-inflammatory, and antitumor effects. Among its antitumor properties, GA inhibits cell proliferation, migration, and metastasis [15] and induces apoptosis [16]. In this study, we assessed the effect of GA on the expression of the CD44 molecule, the anti-apoptotic BCL-2 protein, and EMT markers (ZEB-1 and Snail-1) in gastric CSCs.

Materials and methods

Cell culture

Gastric CSCs, previously isolated and identified from a gastric cancer patient [17–19] were cultured in suspension using DMEM/F12 medium (Gibco, USA) supplemented with 20 ng/ml human epidermal growth factor (EGF) (Biolegend, USA), 1% B-27 supplement (50X) (Gibco, USA), and 1% penicillin/streptomycin.

Cells were plated into non-treated T-25 flasks (Corning, USA) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Assessing the viability of gastric CSCs with various GA concentrations by MTT assay

The cytotoxic activity of GA was assessed using the MTT assay. Gastric CSCs were seeded in non-treated 96-well plates (Corning, USA) at a density of 3×10^4 cells/well in 100 µl of complete culture medium (DMEM/F12 supplemented with EGF, B-27, and penicillin/streptomycin) and incubated for 24 hours. Cells were then treated with different concentrations of GA (20–160 µM; Sigma, USA) for 24, 48 and 72 hours. After treatment, 20 µl of MTT solution (5 mg/ml; Sigma, USA) was added to each well and incubated for 4 hours at 37°C. Subsequently, 100 µl of DMSO (Sigma, USA) was added to dissolve the formazan crystals, and the plates were gently shaken at room temperature for 70 minutes. Absorbance was measured at 570 and 630 nm using a microplate spectrophotometer (BioTek Epoch, USA).

RNA extraction, cDNA synthesis, and Quantitative real-time PCR

RNA extraction, cDNA synthesis, and Quantitative real-time PCR

Gastric CSCs were cultured in non-treated 6-well plates (Corning, USA) at a density of 7×10^5 cells/ml and treated with 60 μM GA for 48 and 72 hours. Total RNA was extracted using TRIzol™ Reagent (Gibco, USA), and cDNA was synthesized using the Easy cDNA Ultra-TM Synthesis Kit (Parstous, Iran). Quantitative real-time PCR (qRT-PCR) was performed using SolisFAST® SolisGreen® qPCR Mix (Solis BioDyne, Estonia). The sequences of the primers are listed in Table 1. qRT-PCR was carried out using a Thermal Cycler (Applied Biosystems, USA). Relative mRNA expression levels were calculated using the 2^−ΔΔCt method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control.

Detection of surface marker CD44 using flow cytometry

Gastric CSCs were cultured in non-treated 6-well plates at a density of 1×10^6 cells/ml and treated with 60 μM GA for 48 and 72 hours. Following trypsinization and washing with phosphate-buffered saline (PBS; Sigma, USA), cells were resuspended in PBS and incubated for 30 minutes in the dark with APC-conjugated mouse anti-human CD44 antibody (BD Biosciences, USA). Cells were then washed three times with PBS and finally resuspended in 800 μl of PBS. Fluorescence intensity was analyzed using a CyFlow Cube6 flow cytometer (Sysmex, Germany).

GA effect on tumor volume in vivo

All animal experiments were performed in accordance with the ARRIVE guidelines 2.0 for reporting animal research. The study protocol was approved by the Specialized Ethics Committee for Working with Laboratory Animals of Birjand University of Medical Sciences (IR.BUMS.AEC). Gastric CSCs (1×10^6 cells/ml) were subcutaneously injected into the right flank of male nude mice (C57BL/6 strain), aged 4–6 weeks (15–20 grams). A total of 18 mice were randomly divided into three groups (n = 6 per group): (1) CSCs + GA treatment group, (2) CSCs without GA control group, and (3) Matrigel-only group without cells. Cells were suspended in serum-free DMEM (Sigma-Aldrich, USA) containing GA (120 μM) mixed with Matrigel (1:1 ratio; Sigma-Aldrich) for the treated group or in DMEM/Matrigel (1:1) for the untreated control group. Each injection used 100 μl delivered with a microsyringe at the North Research Center, Pasteur Institute of Iran. Tumor growth was monitored weekly, and tumor volume (mm^3) was calculated using the formula: $0.5 \times D_1^2 \times D_2$, where D_1 and D_2 represent the width (larger diameter) and length (smaller diameter) of the tumor, respectively. For humane endpoint, tumor-bearing mice were euthanized by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (10 mg/kg) followed by placement in a desiccator to induce death by hypoxia before recovery from anesthesia.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Data are presented as mean ± SD (n = 3). MTT assay results were compared using one-way analysis of variance (ANOVA), and paired t-tests were used to assess differences in mRNA expression levels. A p-value ≤ 0.05 was considered statistically significant.

Results

GA inhibits growth of gastric CSCs.

The inhibitory effects of GA on gastric CSCs were evaluated using the MTT assay. Our results demonstrated that GA inhibited cell proliferation in a dose- and time-dependent manner (Fig. 1). The IC_{50} values of GA for gastric CSCs were 106 μM at 48 hours and 84 μM at 72 hours. Based on these findings, a concentration of 60 μM GA was selected for subsequent experiments.

GA Reduces Expression Levels of CD44, Bcl-2, and EMT Markers, and Surface CD44 Protein in Gastric CSCs.

Following exposure to 60 μM GA for 48 and 72 hours, the mRNA expression levels of CD44, Bcl-2, and EMT markers (ZEB-1 and Snail-1), as well as the protein expression of CD44, were significantly decreased compared to untreated cells (Figs. 2 and 3).

GA Reduces Tumor Size In Vivo.

Injection of a mixture of gastric CSCs with GA into nude mice resulted in inhibited tumor growth and a reduction in tumor volume compared to the untreated group (CSCs without GA) (Fig. 4).

Discussion

Gastric cancer (GC) is a highly malignant disease with a poor prognosis. Common treatments for GC include surgery and chemotherapy; however, due to tumor recurrence and metastasis, these therapies have not been very effective [20]. CSCs are considered a major cause of the inability to completely eradicate tumors because of their role in tumorigenesis, self-renewal, and pluripotency [21]. Therefore, therapies targeting CSCs are of great importance [22]. Plant-derived active ingredients have been proposed as potential therapeutic agents with possibly fewer side effects compared to conventional chemotherapy drugs [23]. In this study, we used 18β-glycyrrhetinic acid (GA), an active component of licorice root, to investigate its effects on inhibiting CSC growth both in vitro and in vivo. Additionally, we assessed the impact of GA on the expression of CD44, EMT markers, and the anti-apoptotic protein BCL-2 in gastric CSCs.

Many studies have demonstrated the antitumor effects of GA on cancer cells. For example, exposure of colorectal cancer cell lines (LoVo, SW480, and SW620) to GA activated pro-apoptotic pathways and inhibited cell growth [16]. Similarly, GA inhibited the proliferation and induced apoptosis in various hepatocellular carcinoma (HCC) cell lines, including HepG2, SMMC-7721, HLF, HLE, Hep3B, and LM3 [24]. In ovarian cancer cells (SKOV3 and OVCAR3), GA and its derivatives suppressed CSC properties and induced apoptosis [25]. Hsu et al. also demonstrated that GA induced apoptosis in breast cancer cell lines (MCF-7 and T-47D) and inhibited tumor growth in vivo (BALB/c nude mice) [26]. Consistent with these findings, we showed that GA has antitumor activity against gastric CSCs, reducing their growth in vitro (Fig. 1) and in vivo (Fig. 4), and downregulating the expression of the anti-apoptotic gene BCL2.

The cell surface adhesion receptor CD44 is a widely recognized marker for CSCs and plays a key role in regulating both CSC maintenance and the epithelial-mesenchymal transition (EMT) phenotype. Due to its involvement in both upstream and downstream signaling pathways that regulate stemness and EMT, CD44 is considered a valuable target for therapeutic intervention [27]. Thus, blockade or depletion of CD44 in CSCs results in the attenuation of the malignant phenotype, inhibition of cancer growth, and reduced resistance to therapy [28]. For example, overexpression of CD44 in SW480 colon cancer cells leads to the induction of EMT-related changes—such as increased expression of EGFR, activation of PI3K/Akt signaling, and changes in E-cadherin/β-catenin complex formation—whereas knockdown of CD44 results in the downregulation of EMT markers like fibronectin, α-actin, N-cadherin, and MT1-MMP [10].

Accumulating evidence suggests that GA can reduce CD44 expression and attenuate EMT-related changes.

For instance, GA has been shown to upregulate miR-328, which subsequently reduces CD44 expression, thereby impairing cancer cell growth and reducing chemotherapy resistance [29]. Additionally, a GA derivative (compound 7c) was reported to downregulate CD44 expression in ovarian cancer cells [25]. Beyond its effects on CD44, GA influences EMT through several signaling pathways. Aberrant activation of the STAT3 pathway has been associated with the upregulation of EMT-related transcription factors such as ZEB, TWIST, and SNAIL [30, 31]. GA has been shown to reduce STAT3 phosphorylation, thereby suppressing TGF-β-induced EMT [32]. Moreover, GA inhibited the migration of A549 lung cancer cells by modulating ROS/MAPK/STAT3/NF-κB signaling pathways; it simultaneously increased E-cadherin expression while decreasing the expression of N-cadherin, Snail-1, and vimentin [33]. In agreement with these studies, our data demonstrate that GA reduces the expression of CD44 and EMT markers (Snail-1 and ZEB-1) in gastric CSCs (Figs. 2 and 3).

Conclusion

In summary, considering the effects of GA on decreasing the expression of CD44, BCL-2, and EMT markers, along with its ability to inhibit xenograft tumor growth, and given the direct relationship between CD44 expression and EMT changes, it can be concluded that GA likely exerts its inhibitory function through one or more EMT signaling pathways dependent on CD44 expression.

Abbreviations

GC
Gastric Cancer
CSC
Cancer Stem Cell
EMT
Epithelial-Mesenchymal Transition

GA
18β-Glycyrrhetinic Acid
DMEM/F12
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
PBS
Phosphate-Buffered Saline
qRT-PCR
Quantitative Real-Time Polymerase Chain Reaction
GAPDH
Glyceraldehyde-3-Phosphate Dehydrogenase

Declarations

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Competing interests

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.

Authors' contributions

All authors contributed to the study conception and design. Maede sadat rahnamaei: Methodology, Validation, Investigation, Writing - Original Draft. Ramiar Kamal Kheder: Methodology, Investigation, Writing - Original Draft. Omeed Darweesh: Formal analysis, Resources, Writing - Original Draft. Seyedeh Mahya Shariat Razavi: Review & Editing Vahid Bagheri: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. Gholamreza Anani Sarab: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Ethics approval

The current study did not involve new human sample collection. Gastric cancer stem cells were used that had been previously isolated and characterized in an earlier study “Isolation and identification of chemotherapy-enriched sphere-forming cells from a patient with gastric cancer” (Bagheri et al., Journal of Cellular Physiology, 2018), which was conducted under the ethical approval of the appropriate institutional ethics committee. Written informed consent had been obtained from the patient at that time. The Institutional Animal Care and Use Committee of Birjand University of Medical Sciences approved all experimental procedures of this study. The projects entitled “The evaluation of the effects of Glycyrrhetinic acid derived from Glycyrrhiza glabra on the expression of STAT3 related signaling

pathways genes in Gastric Cancer Stem cells" and "Evaluation of the effect of Glycyrrhetinic acid derived from Glycyrrhiza glabra on expression of EMT-related ZEB-1 gene in Gastric Cancer Stem cells" were approved by the Specialized Ethics Committee for Working with Laboratory Animals of Birjand University of Medical Sciences (IR.BUMS.AEC) under project numbers 455983 (Ethics Code: IR.BUMS.REC.1398.353) and 5207 (Ethics Code: IR.BUMS.REC.1398.367), respectively, on February 10, 2020.

consent to participate

Not applicable

Consent for publication

Not applicable

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The authors declare that they have not use AI-generated work in this manuscript

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Table 1

Table 1. Primer sequences for the amplification of target genes and GAPDH

Genes	Primer sequence 5' -> 3'
CD44	For: TCCAACACCTCCCAGTATGACA
	Rev: GGCAGGTCTGTGACTGATGTACA
Bcl-2	For: TGGAGAGTGCTGAAGATTGATG
	Rev: GTCTACTTCCTCTGTGATGTTGT
ZEB-1	For: GTGGCGGTAGATGGTAAT
	Rev: CTGTTTGTAGCGACTGGA
Snail-1	For: TAGCGAGTGGTTCTTCTGCG
	Rev: AGGGCTGCTGGAAGGTAAAC
GAPDH	For: TGGACTCCACGACGTACTCAG
	Rev: CGGGAAGCTTGTCATCAATGGAA

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

Figures

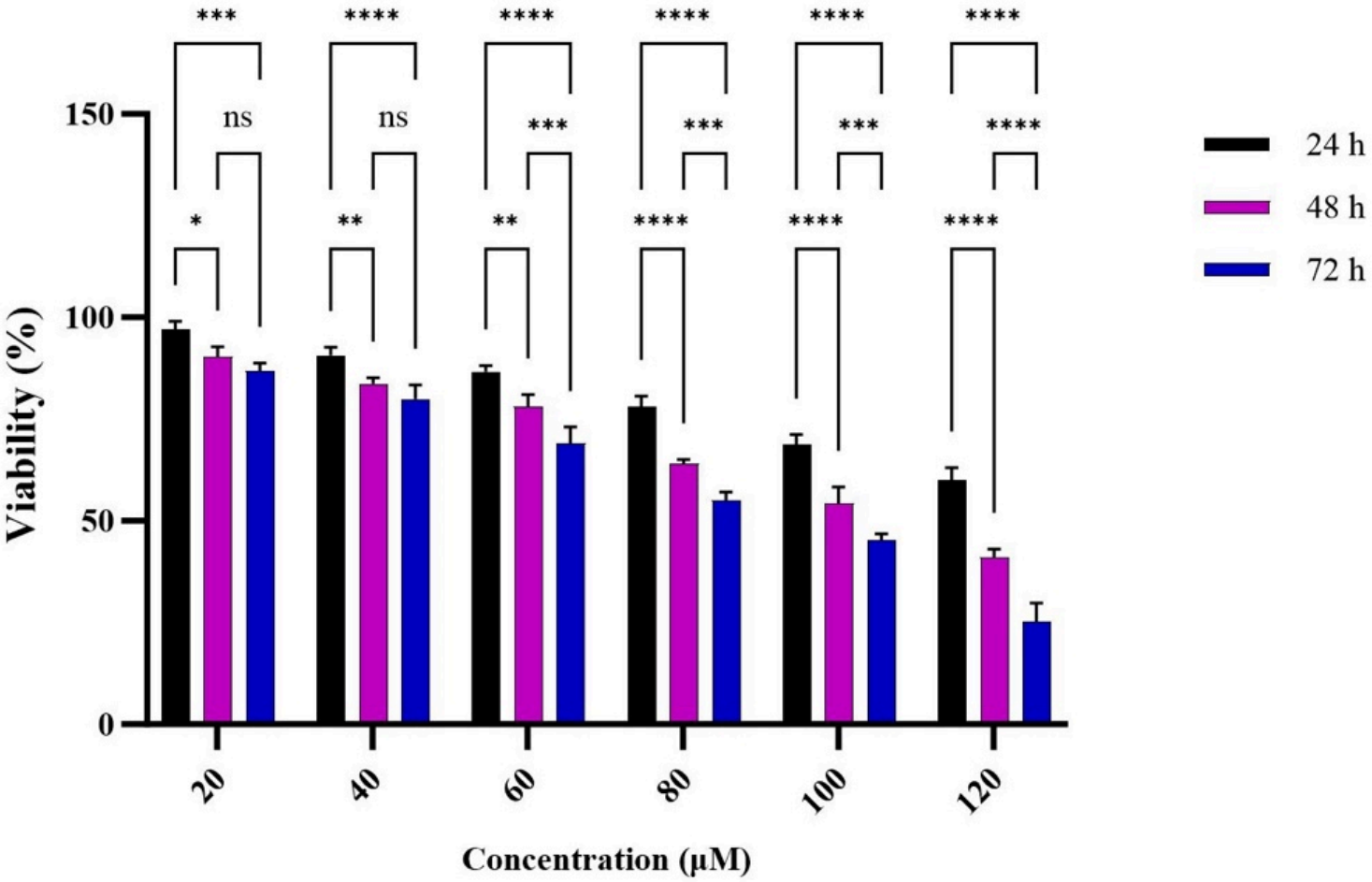


Figure 1

GA inhibited gastric CSCs proliferation. After treating GCSCs with various concentrations of GA (20-160 µM) for 24 h, 48 h, and 72 h, cell viability was significantly reduced in time and dose-dependent manners. The data are represented as mean ± SD. Abbreviations: CSC: gastric cancer stem cells; GA: 18ß-glycyrrhetinic acid.

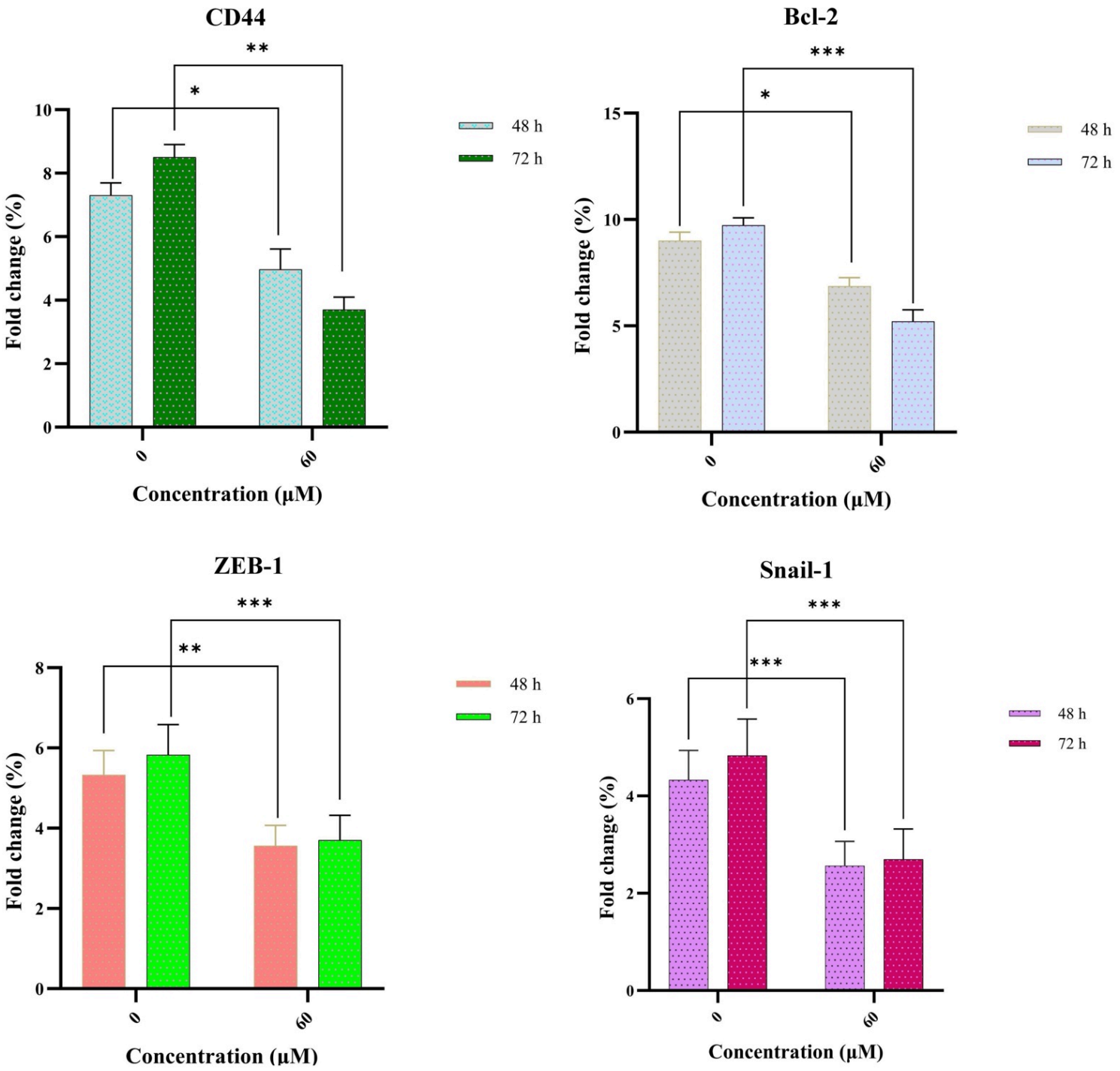


Figure 2

GA reduced mRNA expression levels of genes of CD44, EMT markers (ZEB-1 and Snail-1), and anti-apoptotic protein Bcl-2 in gastric CSCs. The cells treated with a concentration of 60 µM GA for 48 and 72 h significantly downregulated mRNA expression of CD44, ZEB-1, Snail-1, and Bcl-2 in compared with the untreated cells (a). The data are represented as mean ± SD. Abbreviations: GA: 18ß-glycyrrhetinic acid; EMT: epithelial-mesenchymal transition; CSCs: cancer stem cells.

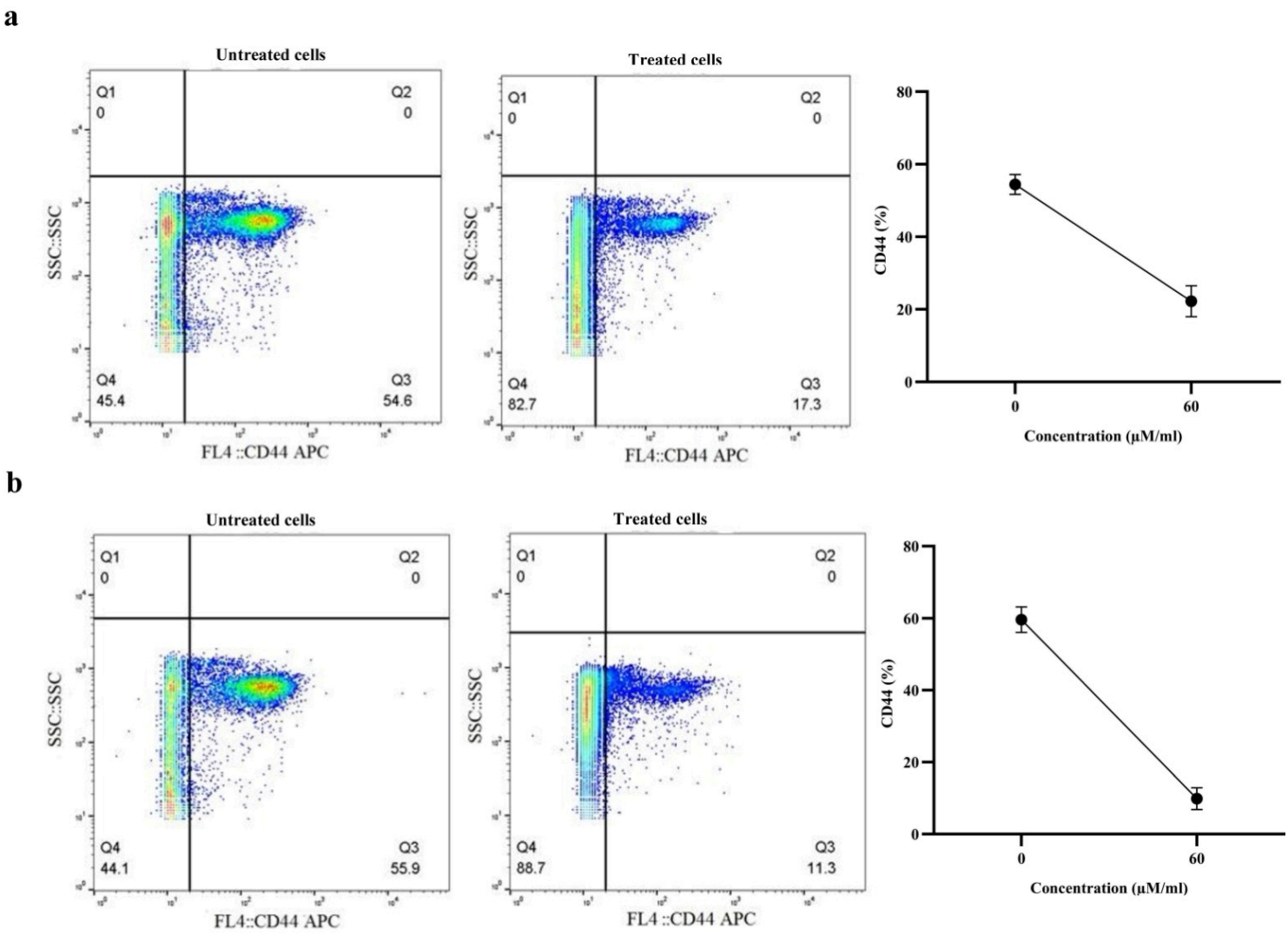


Figure 3

flow cytometry analysis of CD44 expression after treating gastric CSCs with GA. CD44 expression was significantly reduced in the cells after treatment with a concentration of 60 µM GA for 48 (a) and 72 h (b) compared with untreated cells. The data are represented as mean ± SD. Abbreviations: CSCs: cancer stem cells; GA: 18ß-glycyrrhetinic acid.

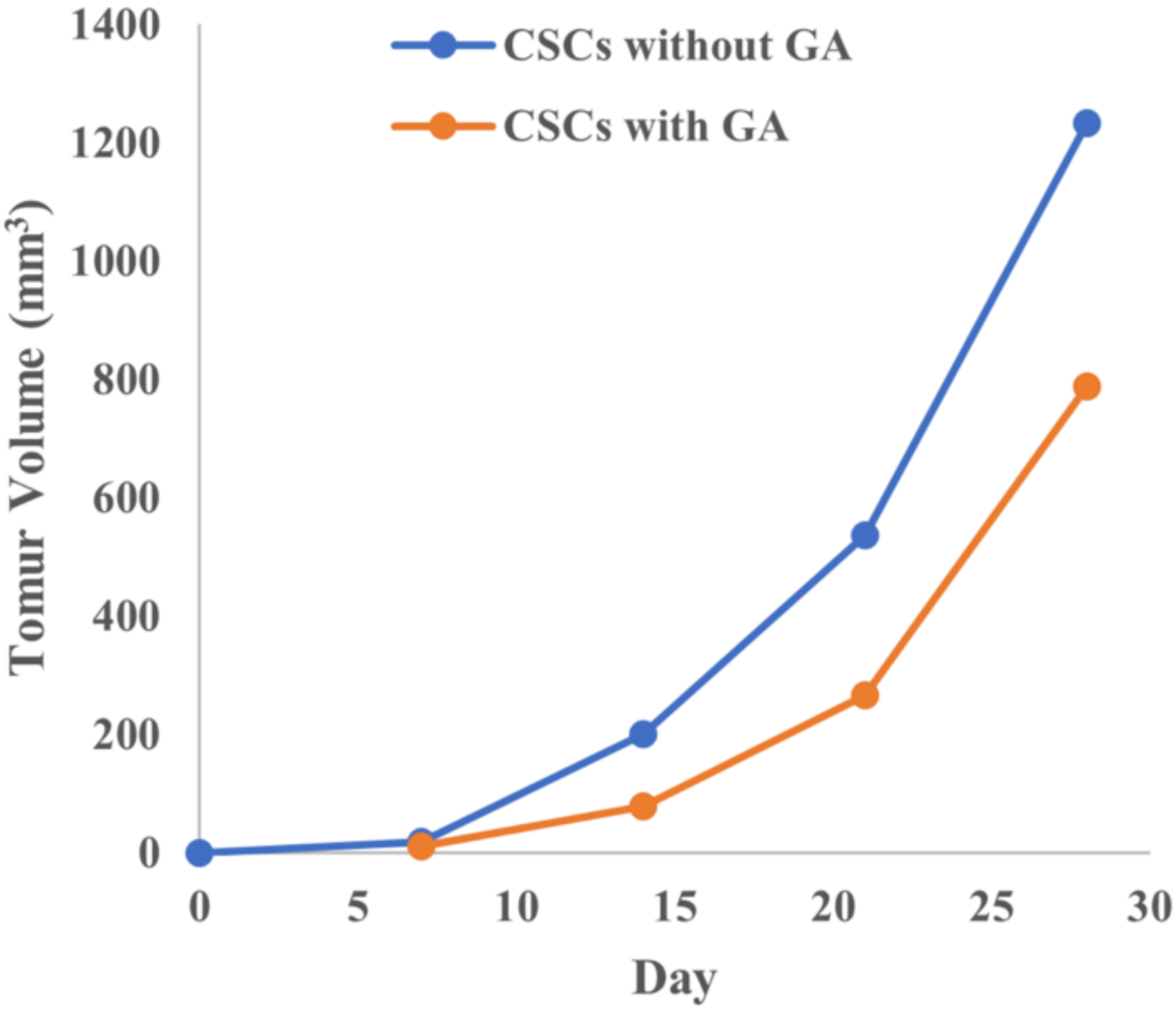


Figure 4

GA inhibited gastric CSCs growth *in vivo*. After inoculation of 1 × 10^6 cells/ml with GA and without GA subcutaneously into the right flank of nude mice, the volume of tumors produced on days 7, 14, 21, and 28 was reduced in treated group (CSCs with GA) compared to untreated group (CSCs without GA). Abbreviations: CSCs: cancer stem cells; GA: 18ß-glycyrrhetinic acid.