Gemini curcumin inhibits 4T1 cancer cell proliferation and modulates the expression of apoptotic and metastatic genes on Balb/c Mice model

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Research Article

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Abstract

Background/aim:

Despite the attractive anticancer effects, poor solubility and low bioavailability have restricted the clinical application of curcumin. Recent findings show that gemini nano-curcumin (Gemini-Cur) significantly improves cellular uptake of curcumin and its anticancer effect in tumor cells. Here, we aimed to assess the suppressive effect of Gemini-Cur on 4T1 breast cancer cells *in vitro* and, subsequentlly on BALB/c mouse models.

Materials and methods

Fluorescence microscopy was employed to visualize cellular uptake and morphological changes of 4T1 cells during treatement with Gemini-Cur and void curcumin. MTT and annexin V/FITC assays were performed to study the toxic effect of Gemini-Cur on mouse cancer cells. For *in vivo* studies, BALB/c tumor-bearing mice were used to evaluate the inhibitory effect of Gemini-Cur in comparison with mice receiving free curcumin and nanoparticles.

Results

Our data showed that Gemini-Cur enters the cells and inhibits proliferation in a time- and dose-dependent manner. Annexin V/FITC confirmed apoptotic effect on 4T1 cells. *In vivo* studies also illustrated that tumor growth is suppressed in Gemini-Cur treated mice rather than controls. Expression studies demonstrated the modulation of apoptotic and metastatic genes including Bax, Bcl-2, MMP-9, VEGF and COX-2 in treated mice.

Discussion

Taken together, these data demonstrate the promising anticancer properties of Gemini-Cur on mice models. However, further studies in molecular and cellular levels are required to conclude this therapeutic advantage.

1. Introduction

Cancer is a multistep disease that is resulted by myriad of deregulated genes and molecular pathways. Breast cancer (BC), as a multiple phenotype malignant tumor, demonstrated to be the second prominent cause of cancer-related mortality among women in the developing countries and the most prevalent form of malignancy diagnosed in developed countries[1]. Cancer metastasis is responsible for about 90% of all deaths from cancer and agents that prevent metastasis provide a meaningful advantage in cancer treatment. Due to shortcomings of common treatments such as hormone therapy, chemotherapy and

surgery specially in advanced stages, there are increasing demands for natural compounds which can confront cancer progression [2].

In recent years, several anticancer compounds with different mechanisms of action have been produced from plant sources [3, 4]. Curcumin is the main component of Curcuma longa L. (turmeric) that has attracted great interest due to its valuable therapeutics properties such as antioxidant, anti-tumor, anti-mutagenic, antimicrobial, and anti-inflammatory activities[5].

Curcumin exhibits unique anticancer activity by inhibiting proliferation and inducing apoptosis. Many studies showed curcumin's antitumor activity on numerous cancers including lung, breast, head and neck, brain, and prostate cancer. However, the application of curcumin is restricted because of its low water solubility and cellular bioavailability [6]. In recent years, the bioavailability and aqueous solubility of curcumin has effectively improved by using nano-based drug delivery systems [7, 8].

Gemini surfactant nanoparticles are a group of surfactant family with two or more hydrophilic head and tail groups linked by a flexible or rigid spacer. Gemini surfactants possess unique characteristics, such as enhanced water solubility, low critical micelle concentration, aggregation behavior, and unusual micelle structures, high efficiency in reducing water/oil interfacial tension, and remarkable rheological properties that have attracted much attention [9]. The spacer in Gemini surfactants has the ability to connect two hydrophilic groups via chemical to form a structure. The current configuration offers more advantages, including higher micelle forming ability, higher surface tension, and better water solubility. Particularly, Gemini surfactants in very low concentrations are enough to form spheroidal micelles and don't have toxicity to the host cells [10].

Recently, our group has formulated gemini curcumin as stable formulation of curcumin with significant antitumor effects. [11, 12]. To further investigate the suppressive characteristics of Gemini-Cur on tumor models, we employed breast cancer models of BALB/c mice through evaluation of tumor growth, apoptosis and metastasis.

2. Materials And Methods

2.1 *In vitro study*

2.1.1. *Cells and reagents*

4T1 cell line, a metastatic breast cancer cell line originated from the mammary gland tissue of a mouse BALB/c strain (was purchased from Pasteur Institute, Tehran, Iran) was cultured using RPMI 1640 (Sigma Aldrich, USA) medium enriched with 10% fetal bovine serum (FBS; Sigma Aldrich, USA), 100U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere providing 5% CO2. Cur and Gemini surfactants were a kind present of Institute for Color, Science and Technology, Tehran, Iran. Gemini-Cur was prepared as mentioned on previous report [11].

2.1.2. *Uptake kinetics*

The cellular uptake and localization of void curcumin (dissolved in PBS) and Gemini-Cur nanoparticles were analyzed using flow cytometry and fluorescence microscopy. The 4t1 cells were seeded onto the sterile glass coverslips placed in a 6-well plate at density of 6×10^4 cells per well containing 2ml RPMI media. After 24-hour incubation, cells were washed with PBS and treated with 30 μM of void curcumin as well as Gemini-Cur for 3 hour. Finally, the cells were rinsed with PBS three times and imaged using fluorescence microscopy (Olympus BX41, Germany). To measure the intracellular curcumin content, cells were washed and assembled with centrifugation at 800 rpm for 5 min. By using flow cytometry the intensity of the interiorized curcumin was recorded.

2.1.3. Cell viability assay

Using MTT assay, cell viability was assessed according to the manufacturer's instructions (Sigma-Aldrich, USA). Briefly, 4T1 cells were loaded onto 96-well plates and permited to adhere and cultivated overnight in 200 μl medium. After, The cells were incubated with fresh medium comprised of consecutive concentrations (0 to 100 μM) of Gemini curcumin and void curcumin solutions for 24 and 48 h. Void curcumin was used as negative control. Afterward, 20 μl of 5 mg/ml MTT (Sigma Aldrich, USA) was added to each well and incubated for additional 4 h at 37°C followed by addition of 100 μl of di-methyl sulfoxide (DMSO). The relative cell viability was measured at 570 nm by an ELISA reader and the half maximal inhibitory concentration (IC50) was measured by standard curve. Each experiment was done in triplicate wells and repeated three times minimum.

2.1.3. *Apoptosis assay*

To determine the mode of toxicity, annexin V/FITC staining was done using flow cytometry pursuant to manufacturer's instructions. 4T1 cells at a density of 2.5×10^4 were loaded into 6-well culture plates and then incubated with 12 μM of Gemini-Cur and void Gemini for 24 h. Using binding buffer cell pellet were cleaned 2 times and then incubated the cells with 10 μl of annexin V-FITC in a dim spot. After 15 min, cells were washed with 1 ml of binding buffer and centrifuged. At last, cell pellet was dissolved in a mixture of 500 μl/5 μl of binding buffer / PI solution prior to analysis by flow cytometry. The experiment was repeated three times.

2.2. In vivo study

2.2.1. Tumor models

Female inbred BALB/c mice at 5–7 weeks of age were bought from Pasteur Institute, Tehran, Iran, and housed (four per cage) under the standard conditions (controlled temperature and humidity) according to the guideline of Animal Care and Research Committee of Tabriz University, consistent with the Guide for the Care and Use of Laboratory Animals. A total of 1×10^6 4T1 cells in logarithmic growth phase were solved in 150 μl of PBS (1×10^6 cells/150 μl PBS) and then, were injected subcutaneously (s.c.) into the right flank of BALB/c mice and allowed to form tumor mass. Once tumor mass became tangible on day

7, animals were randomized in 3 groups (n = 6 per group), including control (without any treatment), Gemini nanoparticles (Gemini NP; received gemini surfactant) and Gemini-Cur. For Gemini Cur, 12.5 mg/kg bw (body weight) of curcumin was injected daily. Tumor volumes (mm3) were sized 4 times per week with the aid of a vernier caliper until sacrificing mice on day 28 post injection and was evaluated by the formula: Length×width^2 × π/6.

2.2.2. Histological assay

In order to confirm the type of tumor, biopsies were processed for hematoxylin and eosin staining and evaluated by an expert.

2.2.3. Real-time reverse transcription-PCR

To evaluate gene expression in tumor models, total RNA was first isolated from tumor masses using BRIzol reagent (Faragene Co., Tabriz, Iran) according to the supplied protocol. The quality and concentration of extracted RNA were evaluated using the NanoDrop spectrophotometer (ThermoFisher Scientific Life Sciences, USA). Then, total RNA was reverse transcribed into complementary DNA (cDNA) with AddScript Enzyme solution (AddScript cDNA Synthesis Kit, Korea) in the presence of random hexamer following manufacturer's instructions. Synthesized cDNA was then subjected to real-time PCR using 2X Real-Time PCR Master Mix (BioFACT, Korea) in the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). To normalize Bax, Bcl-2, MMP9, VEGFA and COX-2 expression, β2 microglobulin was used as internal control. Changes in mRNA expression were shown as fold change. Primers used in this study were presented in Table 1.

2.2.4. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was carried out using the One-way analysis of variance (ANOVA) to compare results. All data were expressed as mean ± S.D. Differences among groups were stated to be statistically significant when P value was less than 0.05. All statistical analyses were conducted with Prism® 7.1 software (GraphPad Software, USA)

3. Results

3.1. Cellular uptake of Cur versus Gemini-Cur

The cellular uptake of free curcumin and Gemini-Cur by 4t1 cells in a time dependent manner was visualized by fluorescent microscopy. Equal concentrations of curcumin in both void and gemini-enriched forms was administered. Control group (untreated cells) is shown as our reference value. As shown in Fig. 1A, Gemini-Cur entered into the cells after a 3hour incubation time. However, free curcumin is detectable as crystals outside the cells (Fig. 1A). To further investigate the intracellular amount of Gemini-Cur, the fluorescence intensity of curcumin was recorded using flow cytometry. Figure 1B shows that Gemini-cur treated cells exhibit stronger fluorescence intensity signals than two other groups when there is an equal count of cells which indicates the higher cellular uptake of Gemini-cur in comparison with two

other groups. As shown in Fig. 1C in comparison to control cells which is considered to be 100, two other groups have lower intensity which indicates that there is no fluorescence intensity in Cur and Gemini-Cur groups in negative cells. But in positive cells intensity is significantly ($p < 0.001$) increased in Gemin-Cur treated cells comparing with Cur treated and control group which means high cellular uptake and subsequent elevated fluorescence intensity.

3.2. Effects of Gemini-Cur on *4T1* cell viability

As illustrated in Fig. 2, curcumin decreased 4T1 cell proliferation compared to control in a dose and time-dependent manner. However, loading of curcumin on Gemini surfactants NPs suppressed cell viability more effectively than curcumin alone. The IC50 was reduced from 82.58 μM and 59.93 μM in curcumin treated group to 44.97 μM and 22.09 μM in Gemini-Cur through 24 and 48 h incubation, respectively; indicating the improvement of anti-proliferative effect of curcumin.

3.2. Gemini-Cur induces apoptosis in *4T1* cell line

To determine whether the toxicity of curcumin results from apoptosis, 4T1 cells were incubated with different Gemini-Cur and void Gemini. Staining the cells with annexin V and propidiumiodide (PI) can differentiate apoptosis from other common deaths such as necrosis. As shown in Fig. 3, despite no significant difference between control and void Gemini NPs, Gemini-Cur was able to remarkably ($p < 0.0001$) induce apoptosis in 4T1 cells compared to control cells, indicating that Gemini-Cur exert anti-proliferative effects through apoptosis induction.

3.3. Visual observations

Through *in vitro* investigations, although animals' weights were raised in all control, nano-treated and Gemini-Cur groups up to third week, there were no significant Body weight differences. After 3 weeks, we observed a slight decrease in all groups, which is anticipated due to tumor development. Except for a slight drop in food intake (g/day), there were no significant difference in terms of food intake between groups. There were no behavioral changes in the animals during the period of administration, or in the follow up time.

3.4. Tumor incidence and metastasis

Our observation illustrated that tumor incidence in all groups was 100%. As illustrated in Fig. 4, tumor size in control and Void Gemini nanoparticles treated mice was remarkably more than Gemini-Cur treated group. The obtained results showed that the average tumor size was significantly ($p < 0.0001$) reduced in Gemini-Cur treated group compared to control and Void Gemini NPs treated cells, 17, 19 and 21 weeks after injections.

3.5. Gene expression Analysis

To further confirm the obtained results, qRT-PCR analysis was done to measure the gene expression patterns in treatment groups of mice models. As shown in Fig. 5, Bcl-2 expression levels were significantly ($p < 0.0001$) downregulated Gemini-Cur treated mice models (12.5 mg/kg) in comparison to Void Gemini treated group and control. Besides, pro-apoptotic gene of Bax was significantly ($p < 0.0001$) upregulated in Gemini-Cur treated group (12.5 mg/kg). Also, treatment of breast cancer mice models with Gemini-Cur led to significant ($p < 0.0001$) downregulation of COX-2, VEGFA and MMP9 at mRNA levels in tumor masses compared to void Gemini-treated and control groups.

4. Discussion

Despite its therapeutic significance in multiple human cancers, curcumin-based treatments suffer some drawbacks, including its inadequate bioavailability, low-aqueous solubility, and poor absorption, demanding new strategies for improvement of the cytotoxic effects of curcumin [13]. Subsequently, nowadays, biodegradable polymeric nanomaterials, because of some advantages such as bioavailability, stability, and controlled release are suggested to develop new delivery systems and improve effectiveness of curcumin as a hydrophobic component [14]. Therefore, the current study was aimed to examine the improvement of curcumin on breast cancer growth and metastasis trough loading on Gemini surfactant NPs.

The obtained results from in vitro analysis illustrated that loading of curcumin on Gemini NPs increased not only the cellular uptake but also inhibitory effect of curcumin on 4t1 breast cancer cell growth and proliferation. Further in vitro analysis illustrated that Gemini-Cur also led to a reduction in tumor incidence, size and weight in breast cancer models. To understand the mechanism underlying anti-proliferative effect of Gemini-Cur, flow cytometry analysis was performed. Annexin V/PI staining results implied that Gemini-Cur inhibited in vitro and in vivo breast cancer growth through apoptosis induction. Moreover, qRT-PCR results demonstrated that Gemini-Cur induces apoptosis in breast cancer cells through modulating the expression of important member of the Bcl-2 family genes, including Bax and Bcl-2 expression. Deregulated expression of these genes blockades the programmed cell death and promotes tumor growth[15]. Subsequently, Bcl-2 expression level, as an important pro-survival gene that prevents cells from apoptosis induction, was downregulated in treatment group compared to the control, while pro-apoptotic gene of Bax that is involved in release of cytochrome c activating caspase cascade during apoptosis, showed higher expression levels in comparison with control.

Metastasis is considered the leading cause of breast cancer mortality. This process is consequence of tumor cell ability to invade and migrate to other organs[16]. In particular, 70–80 percent of breast cancer cases suffers from invasive form malignancy having potential to metastasize to neighboring and distance body organs through the bloodstream and lymphatic system, which is main cause of breast cancer related deaths[17]. Considering that, in the current study, the effectiveness of designed NPs on anti-metastasis effects of curcumin was also investigated. In vivo histological results established that appearance of invasive tumors in treatment groups was remarkably lower than the control group. To further confirm anti-metastasis effect of Gemini-Cur on breast cancer, the expression of MMP9 was also

measured through the study. qRT-PCR showed that treatment with Gemini-Cur led to a reduction in MMP9 expression in BC tumors. MMP9, as an important matrix metalloprotease, is upregulated through breast tumorigenesis which is correlated with poor survival of patients. MMP9 is capable to degrade basement membrane collagens, leading tumor cells to invade, migrate and metastasize[18]. Consistent with our results, Na Mo and colleagues have previously reported that exposure to curcumin inhibited breast cancer cell invasion through downregulation of TGF-β1-stimulated MMP-9 expression [19]. Furthermore, it was illustrated that endosomal curcumin could reduce MMP9 expression and inhibit invasive tumor appearance in breast cancer mice models [20]. Therefore, it was suggested that Gemini-Cur could also show anti-metastasis effect on breast cancer cells through regulating MMP9 expression.

Angiogenesis through the formation of new vascular networks is a key mechanism to accomplish excessive need of tumor cells for oxygen and nutrients, supporting their rapid and uncontrolled growth and proliferation [21]. Consequently, the expression levels of Vascular endothelial growth factor A (VEGFA), as important modulator of angiogenesis were investigated in the present study. VEGFA is upregulated through tumorigenesis of multiple cancers, including breast cancer which is correlated with aggressive from of malignancy [22]. Besides, VEGFA has been illustrated to provoke stemness, invasion and metastasis in breast cancer cells through modulating mesenchymal markers, including fibronectin, vimentin and N-cadherin [23]. Therefore, targeting VEGFA, as an angiogenic factor involving in initiation and progression of tumor neovascularization is considered a promising option for treatment of several malignancies such as breast cancer [24]. Interestingly, we also found that treatment of breast cancer mice models with Gemini-Cur reduces VEGFA expression in comparison with controls, indicating the significance of designed Curcumin loaded-NPs in suppression of angiogenesis as well. To support this hypothesis, Thulasiraman and colleagues established that curcumin, in addition to increasing the sensitivity of triple negative breast cancer cells to retinoic acid, could reduce VEGFA expression levels [25]. Moreover, curcumin was shown to induce apoptosis *in vitro* and to suppress *in vivo* tumor growth and angiogenesis in breast cancer models via modulating NF-κB signaling pathway which is involved in the regulation of VEGF expression [26, 27]. Therefore, it could be concluded that Gemini-Cur may also exert suppressive effects on angiogenesis of breast cancer cells.

Finally, the effect of Gemini-Cur on Cyclooxygenase 2 (COX-2) expression, as an important factor participating in human breast cancer angiogenesis and lymph node metastasis [28], was examined in treatment groups. The results confirmed that treatment of breast cancer models with Gemini-Cur could effectively downregulate COX-2 expression. COX-2 was previously established to play important part in VEGF-induced angiogenesis through activation of JNK and p38 kinase pathways in vascular endothelial cells[29]. Also, Ki Won Lee and colleagues reported that in human breast epithelial cells curcumin could suppress COX-2 and MMP-9 via inhibition of ERK1/2 phosphorylation and trans-activation of NF-kB [30]. Furthermore, Curcumin has been shown to repress the development of several types of human malignancies by decreasing the production of inflammatory mediators, including COX-2 [31]. Therefore, considering the aforesaid evidences, Gemini-Cur was suggested to induce its suppressive effects on migration and angiogenesis through downregulating COX-2 expression, as a major regulator participating in tumor development.

In conclusion, the results of current study illustrated that loading of curcumin on Gemini surfactants NPs increased its anti-proliferative effect on breast cancer cells through apoptosis induction. This effect was accomplished by modulating the expression of major cell survival regulators including, Bax and Bcl-2. Furthermore, the obtained results showed that Gemini-Cur could effectively inhibit tumor growth in breast cancer mice models. Besides, suppressive effect of Gemini-Cur on metastasis and angiogenesis via regulating MMP9, VEFGA and COX-2 expression was illustrated through current study. Therefore, it is suggested that Gemini surfactants NPs could be considered as an effective strategy for better delivery of curcumin, improving its anti-tumor effects on breast cancer cells.

Declarations

Conflicts of interests

All authors declare that there are no conflicts of interests.

Author contribution

Behzad Zaker Kandjani performed experiments and data analysis and wrote manuscript. Farzam Sheikhzadeh Hesari contributed to carry out the experiments and interrupted the results. Esmaeil Babaei designed, supervised, conducted and wrote the manuscript.

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Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Medical Ethical Committee of the University of Tabriz (IR.TABRIZU.REC.1398.33).

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Tables

Table 1. The sequences of primer used in qPCR.

Target	F & R	Sequence
β2	Forward	5´-ACTGGGATCGAGACATGTG-3´
	Reverse	5´-AGAAGGTGATGTGTACATTGC-3´
MMP9	Forward	5´-TTCCAGTACCAAGACAAAGCC-3´
	Reverse	5´-CACGGTTGAAGCAAAGAAGG-3´
COX-2	Forward	5´ TGAAGAACTTACAGGAGAGAAGG-3´
	Reverse	5´-AGTATTGAGGAGAACAGATGGG-3´
VEGF	Forward	5´-TGAACTTTCTGCTCTCTTGGG -3´
	Reverse	5´-GCTTCGCTGGTAGACATCC-3´
Bax	Forward	5´-CAGAGGATGATTGCTGACG-3´
	Reverse	5´-AAGGTAGAAGAGGGCAACCAC-3´
Bcl-2	Forward	5´-CAGAGATGTCCAGTCAGC-3´
	Reverse	5´-CTCAAAGAAGGCCACAATC-3´

Figures

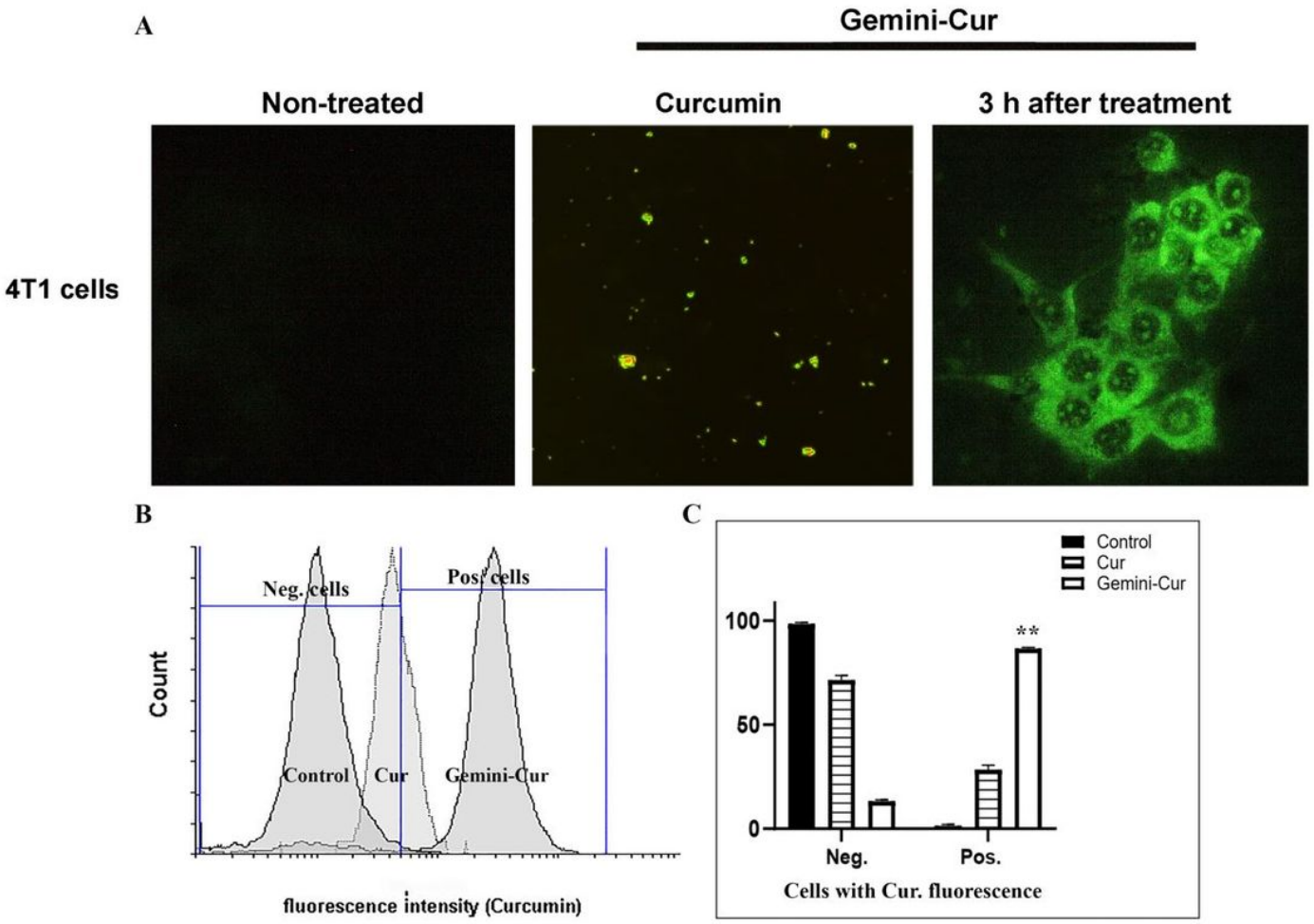

Figure 1

A:Fluorecent microscopic visualization of Gemin-Cur in 4t1 cell line.In comparison to void curcumin treated cells,incororation of Gemini-Cur into cell nucleus is depicted. B: Flow cytometric assessment of cellular uptake of Gemini-cur by 4t1 cells. In treated cells innate fluorescent intensity of curcumin has been evaluated. As shown in an equal cell count the fluorescent intensity of cells treated with Gemini-Cur is higher. C: Diagram of treated cells and control and their fluorescent intensity.

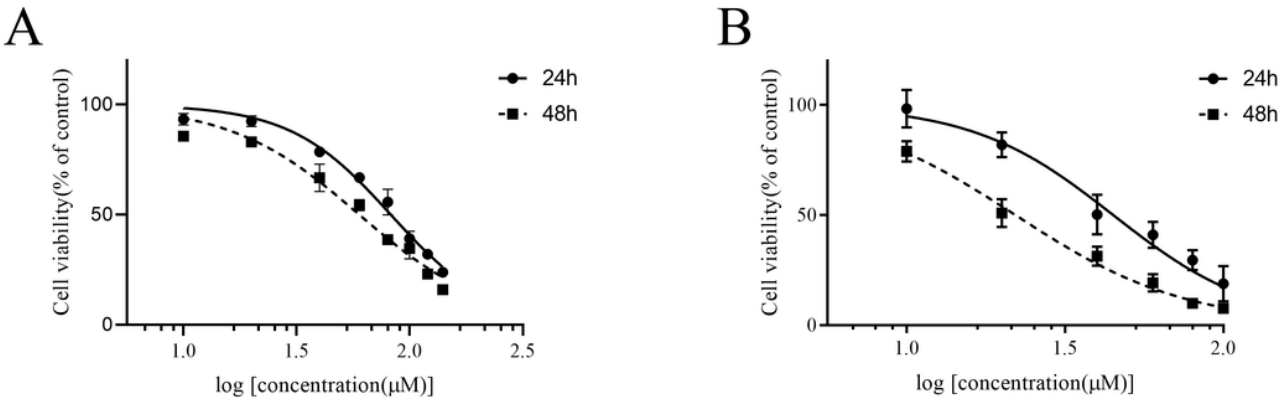

Figure 2

Cytotoxic effects of A: void curcumin and B: Gemini-Cur on 4T1 breast cancer cells.

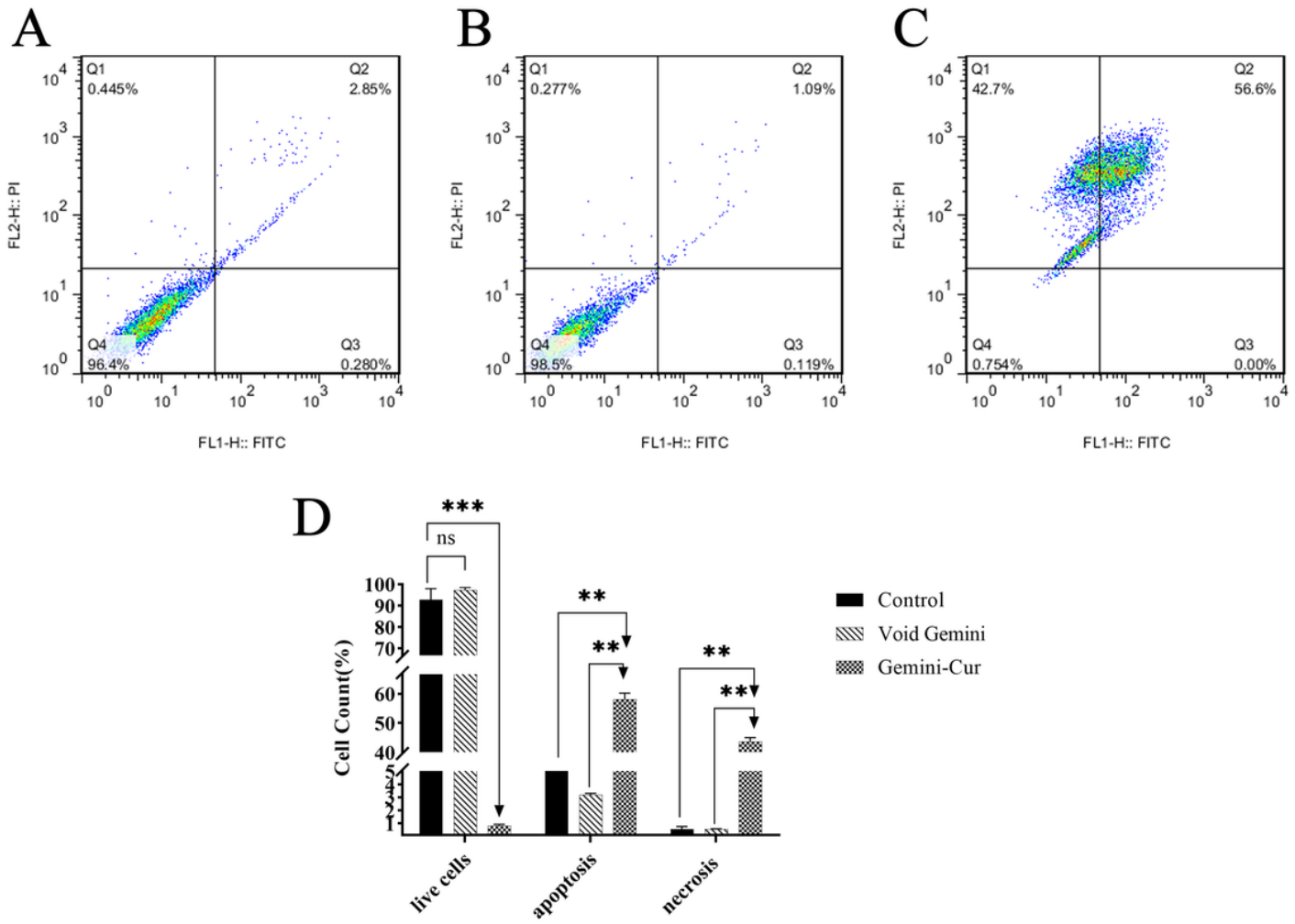

Figure 3

Study of apoptosis by annexin V/FITC. A: 4t1 cells labeled with Annexin V/FITC and no treat(control) B: treated with void Gemini and C: Gemini-Cur treated. Gemini-Cur induced apoptosis in 4T1 breast cancer cells. ****: $p \leq 0.0001$.

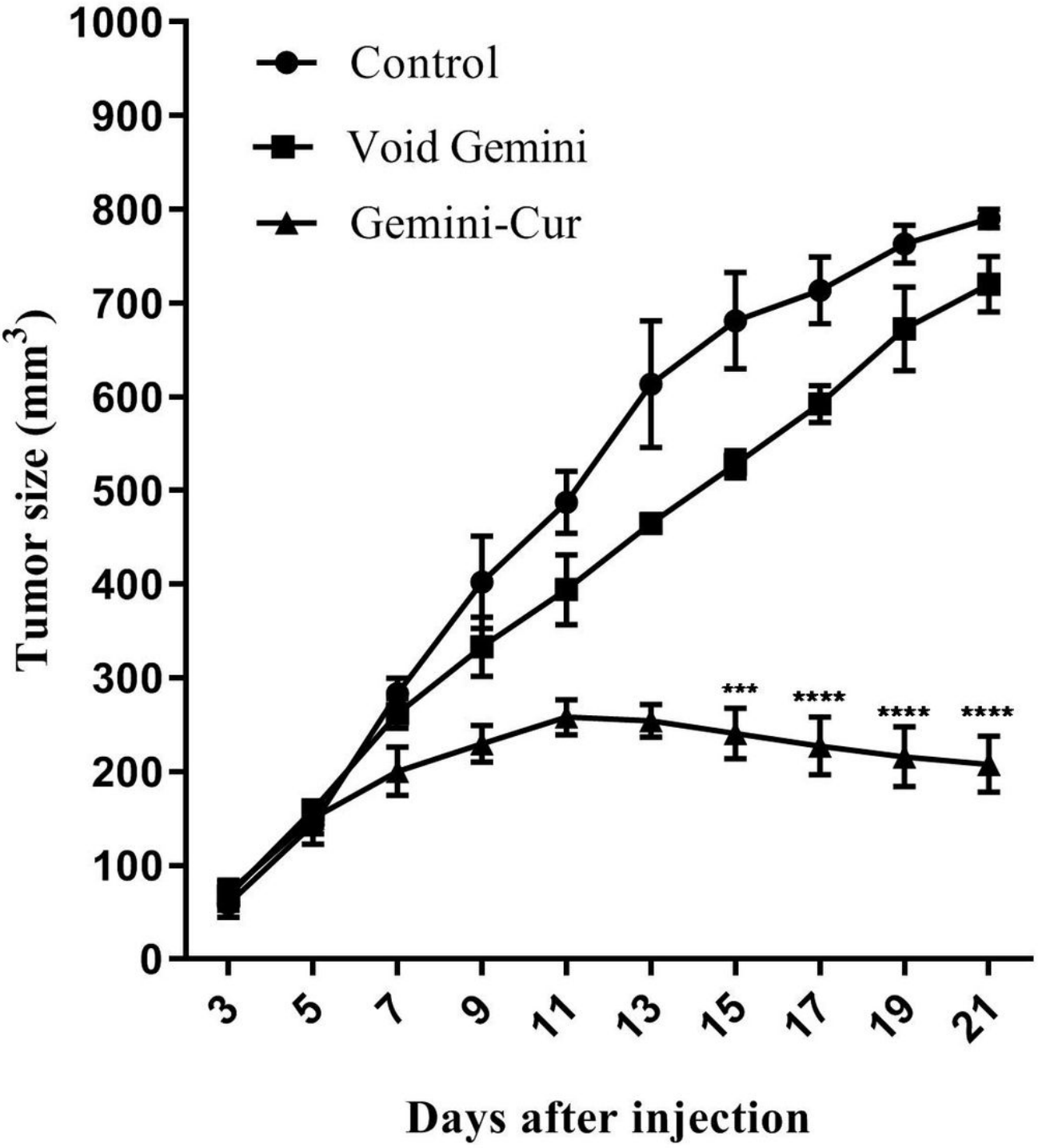

Figure 4

The effect of Gemini-Cur on breast tumor growth. The tumor size was evaluated in different time points after injection of void Gemini NPs and Gemini-Cur compared to control group.

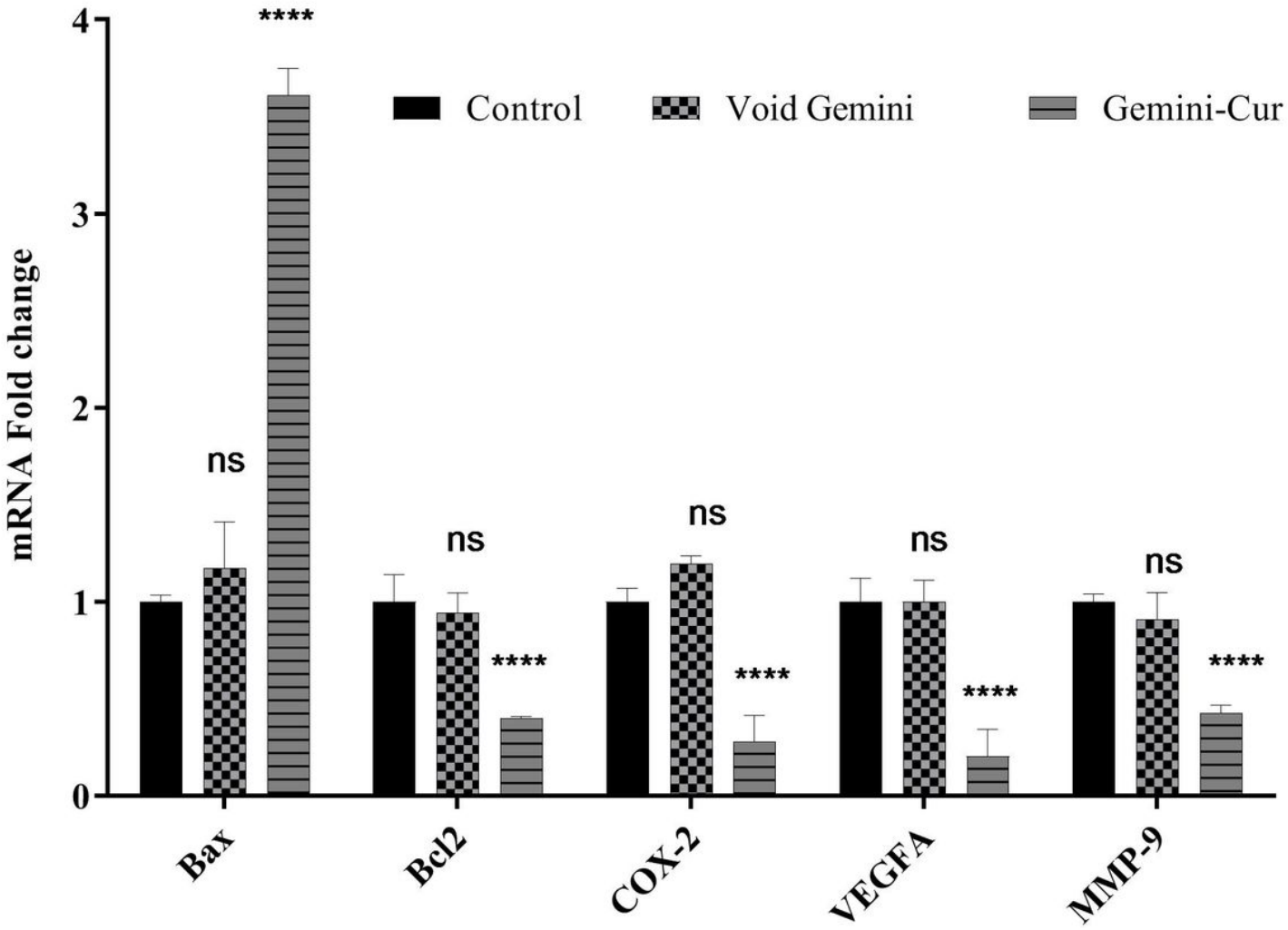

Figure 5

Effect of Gemini-Cur on expression patterns of MMP-9, VEGF, COX-2, Bcl-2 and Bax genes in an animal models of breast cancer. ****: $p \leq 0.0001$, NS: non- significant.