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Association of Galectin-3 but not Laminin in Tamoxifen-Induced Growth Suppression in Breast Cancer MCF-7 Cells

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Research Article

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Abstract

Tamoxifen (TAM), a selective estrogen receptor modulator, competes with estrogen for binding to estrogen receptor (ER) sites, thereby inhibiting estrogen-induced proliferation in ER-positive breast cancer cells. However, long-term treatment with TAM often leads to resistance, and its mechanisms, particularly involving biochemical markers, remain unclear. This study investigated the effects of TAM (10^{-6} M and 10^{-8} M) compared to 17β-estradiol (10^{-6} M and 10^{-8} M) on galectin-3 and laminin expressions in ER-positive MCF-7 breast cancer cells. The MTT assays showed that 10^{-6} M TAM significantly reduced cell viability and altered cellular morphology. Indirect immunofluorescence microscopy revealed that galectin-3 was present on the cell surface and in the nucleus; however, its localization was diminished after 48 hours of TAM (10^{-6} M) exposure. Western blot analysis showed no change in galectin-3 expression at 24 hours but a significant reduction at 48 and 72 hours. In contrast, laminin expression remained unchanged. ELISA results further confirmed reduced galectin-3 but not laminin levels after 48 hours of TAM treatment. These findings suggest that galectin-3, but not laminin, is involved in TAM-induced suppression of MCF-7 cell growth and may serve as a biochemical marker for TAM responsiveness.

Keywords: MCF-7 Cells; Western Blot; Fluorescence Microscopy; Galectin-3, Laminin

Abbreviations

TAM: Tamoxifen; ER: Estrogen Receptor; ECM: Extra Cellular Matrix; FBS: Fetal Bovine Serum

Introduction

Galectins are a family of nonintegrin β-galactoside-binding lectins with related amino acid sequences [1]. Galectin-3, formerly known as CBP35, Mac-2, and εBP, because of its affinity for IgE and HLB31 and its relationship with laminin. It is a 31 kDa-galactoside-binding lectin and a member of the galectin family. Galectin-3 is widely involved in tumor cell migration, adhesion, differentiation, and metastasis [2,3]. Galectin-3 has an anti-apoptotic role and high expression level in breast cancer cells [4]. Although the precise role of galectin-3 remains to be determined, several studies have shown that the expression of galectin-3 is positively correlated with the metastatic potential of several tumorigenic cell lines.

However, the generality of these findings about epithelial cell-derived human tumors is unclear, as increases and decreases galectin-3 have been reported during the malignant

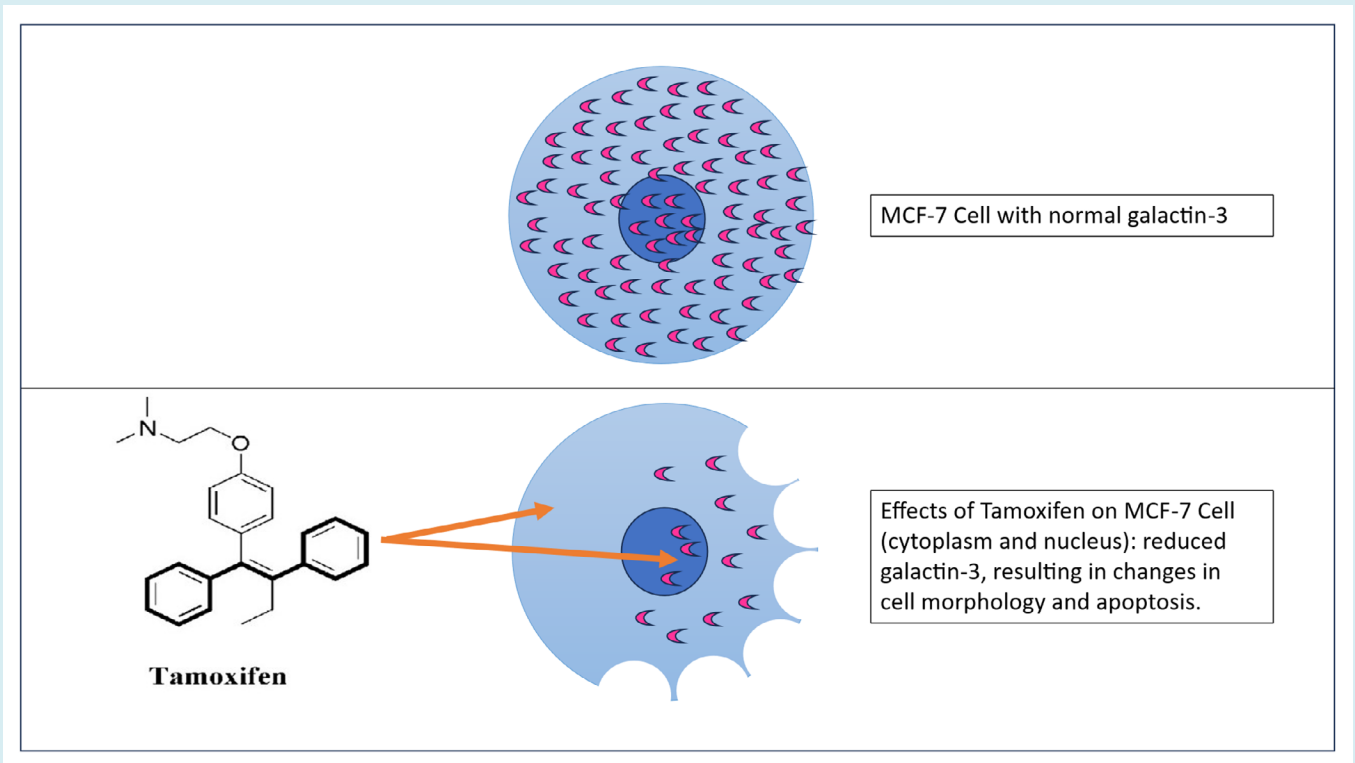
progression of several cancers [5]. Thus, its regulation is critical as an underlying pathway in estrogen-responsive cells when exposed to estrogens or anti-estrogens.

Therefore, we have studied the expression of galectin-3 in MCF-7 cells following treatment with 17β-estradiol (E_2) and tamoxifen (TAM).

In addition, we have tested laminin expression in MCF-7 cells under the same treatment regimen because cell-matrix interaction is considered one of the most significant advances in cancer research [6]. The non-cellular extra-cellular matrix (ECM) component, laminin, is a complex structure involved in cellular adhesion and differentiation in breast tumor development by influencing the surrounding cells. Moreover, the interactions between intracellular protein, galectin-3, and ECM protein laminin are possible through their binding cascade and overall cytoskeletal architecture. This interaction mechanism facilitates signaling pathways. Therefore, we tested both galectin-3 and laminin as biochemical markers in this study.

It has long been recognized that tamoxifen can bind to the ER receptors, thereby acting as a competitive inhibitor to estradiol [7,8]. It attaches ERs to tumors and other tissue targets, producing a nuclear complex that decreases DNA synthesis and initial estrogen effects [9]. Thus, tamoxifen reduces metastasis and lowers the risk of death from breast cancer [10]. One mechanism in which anti-estrogens such as tamoxifen are believed to function is by holding the dividing cell in the G1 phase of the cell cycle, thereby decreasing cell proliferation [11,12]. The role of galectin-3 protein in cells in programming cell death in response to tamoxifen or other hormonal treatments is unknown. In one 72-hour analysis, it was determined that for MCF-7 cells, mRNA and protein levels associated with ER expression were slightly elevated or unregulated with very low concentrations of 10^{-10}M and 10^{-11}M estradiol. In contrast, higher concentrations reduced mRNA and protein expression over the 72-hour study period [13].

This study examined the effects of tamoxifen and its counterpart estradiol over 24-72 hours of exposure. We tested their results one day earlier, and the nature of these effects on the MCF-7 cell cycle regarding anti-proliferative and growth suppressive response to tamoxifen with a concomitant change in galectin-3 or ECM component laminin. Currently, there is no report examining the relevance of galectin-3 or laminin to the short-term treatment of tamoxifen in ER-positive breast cancer cells. Our study demonstrates that tamoxifen-induced growth suppression and death of MCF-7 breast cancer cells are associated with galectin-3 downregulation while laminin levels remain unaltered.



A mechanistic model depicting galectin-3 downregulation due to tamoxifen-induced cellular changes.

Materials and Methods

Cell Culture

MCF-7 human mammary adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and routinely cultured in Dulbecco's Modified Eagle's/ F-12 media (DMEM; Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C. After trypsinizing and counting, all cells were maintained in phenol red-free growth medium supplemented with 10% charcoal-stripped FBS to evaluate the effects of the treatments.

For western blotting, cells (1.0×10^6 cells) were cultured on eight 60 mm plates for 24 hours in phenol red-free medium supplemented with 10% charcoal-stripped FBS at 37°C in 5% CO_2 and cultured in the same medium for 24 to 72 hours in the presence and absence of the treatments as mentioned below.

For flow cytometry, 5.0 x 10^5 cells were plated in duplicate on eight 35mm plates. Typically, after 24 hours of incubation, growth media was replaced with 10% charcoal-striped FBS-containing phenol red-free medium (control), media containing 0.01% DMSO (as vehicle control), or media containing E_2 (10^{-6} M, 10^{-8} M), and TAM (10^{-6} M, 10^{-8} M). We have monitored the cell culture conditions routinely under a Nikon Phase-contrast microscope.

MTT Assay

Cell viability was determined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [14]. This assay followed instructions in the MTT assay kit from Sigma-Aldrich (St. Louis, MO, USA).

M-PER Protein Extraction

After 24-72 hours of in vitro exposure to the MCF-7 breast cancer cell line, the media were removed from the culture dish, and cells were thoroughly washed with 1X phosphate buffer saline (PBS). 1X HALT protease inhibitor cocktail was added to the mammalian protein extraction reagent (MPER), and 200µl of the cocktail was added to the culture dish, which was then placed on ice. After 20 minutes, the cells were centrifuged for 10 minutes at 200 x g. The supernatant was collected in microfuge tubes, and the solubilized protein samples were stored at -80ºC.

Protein Assay

Cells were removed from the culture dishes, rinsed with PBS, and collected into plastic tubes. The pooled cells underwent sonication and were then collected in PBS by centrifugation (@ 200 x g for 5 min). Protein samples were stored at -80°C until assayed. The BCA protein assay kit was purchased from Pierce Chemical Company (Rockford, Ill) and used to determine cell protein concentrations as described earlier [15].

ELISA

Fifty microliters of protein samples were added to each well of a 96-well culture plate and incubated overnight. We washed three times with a wash buffer before blocking for 1 hour. Then, we added 50 µl of rabbit polyclonal galectin-3 primary antibody (1:500) and incubated for 1 hour. After washing multiple times, we added goat anti-rabbit secondary antibody (1:5,000) and incubated for 1 hour. We repeated the washing steps numerous times before adding 100 µl/ well of HRP-substrate solution (Bio-Rad Laboratories, Richmond, CA) for 1 hour. Finally, we stopped the reaction by adding 100 µl of 5% SDS solution for 30 min. The wells were read at 415 nm wavelength using the iMark Bio-Rad plate reader. All antibodies from Invitrogen were purchased from ThermoFisher Scientific, USA.

Flow Cytometry

For flow cytometry, 5.0 x 10^5 cells were plated in duplicate on 35mm dishes. After 48 hours of incubation, cells were briefly trypsinized (trypsin-EDTA) and centrifuged at 3500 rpm for five minutes. The cell pellet from each treatment group was fixed in cold ethanol for 15 min and stained with 1mL Propidium Iodide (PI) solution (10 mM Tris-HCl, 10 µg/mL RNase, 10mM NaCl, and 0.1 mg PI/mL and 2.0% NP-40) before analyzing the DNA ploidy distributions at 488 nm wavelength using a Beckman Coulter FACScan flow-cytometer equipped with a 15mW air-cool laser. Nuclear DNA content was examined based on the procedure as described earlier [16].

Fluorescence Microscopy

Cells were grown for 48 hours on a square 22 x 22 mm coverglass in the respective treatments. Cells were then washed in phosphate-buffered saline (PBS), pH 7.4, and were fixed in 3.0% paraformaldehyde solution for 10 minutes, followed by washing twice in PBS containing 0.1% Tween-20. Cells were then incubated for 5 minutes at room temperature with 5% bovine serum albumin in PBS with 0.1% Triton-X-100. Five microliters of galectin-3 rabbit polyclonal antibody were dissolved into 200µl of PBS for each coverslip for 30 minutes at room temperature. This is followed by staining with FITC-conjugated goat anti-rabbit secondary antibody for 30 min. During incubation, coverslips were kept in a covered container to prevent

evaporation. The stained cells were washed twice with PBS, mounted with Prolong antifade mounting medium (Molecular Probes, Eugene, OR), and viewed under a Zeiss fluorescence microscope. All primary and secondary antibodies from Invitrogen were purchased from ThermoFisher Scientific, USA.

Gel Electrophoresis and Western Blot Analysis

Twenty microliters of protein samples from each treatment were loaded in each lane. The proteins were separated by 10 % SDS-PAGE at 100 volts (V) for about an hour, after which the gel was removed. Proteins from the gel were transferred at 200 V to Hybond-ECL nitrocellulose membranes (Pharmacia Biotech, Buckinghamshire, UK) and blocked for 1 hour at 4˚C in 1% Blotto non-fat dry milk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Membranes were incubated with 0.5 µg/ml rabbit polyclonal galectin-3 or laminin primary antibody for 1 hr at room temperature and subsequently in 1 µg/ml peroxidase-conjugated anti-rabbit IgG (Fab specific) at room temperature. ECL western blotting detection analysis (Amersham Biosciences, Buckinghamshire, UK) was used to visualize protein expression on X-ray film (Eastman Kodak Co., Rochester, NY). All antibodies from Invitrogen were purchased from ThermoFisher Scientific, USA.

Statistical Analysis

Experiments were repeated three times with two dishes per treatment, and the mean and standard error (SEM) were calculated. Differences between the various treatment groups were analyzed by one-way repeated measures analysis of variance. Multiple comparison tests analyzed the groups with significant differences ($p<0.05$). Prism 9.02 software (Graphpad Inc., San Diego, CA) was utilized to analyze the data.

Results

Effects of Tamoxifen on Cell Morphology

Representative phase-contrast photomicrographs of cultured MCF-7 cells are presented in (Figure 1). MCF-7 cells incubated for 48 h in 0.01% DMSO (A) appeared morphologically normal in culture (Figure 1A). Cell-cell contacts remained undisturbed. In contrast, treatments with 10^{-8} M (Figure 1B) altered cellular morphology. In particular, MCF-7 cells were retracted and shrunken with prominent cytoplasmic extensions when exposed to TAM compared to the control (Figure 1A).

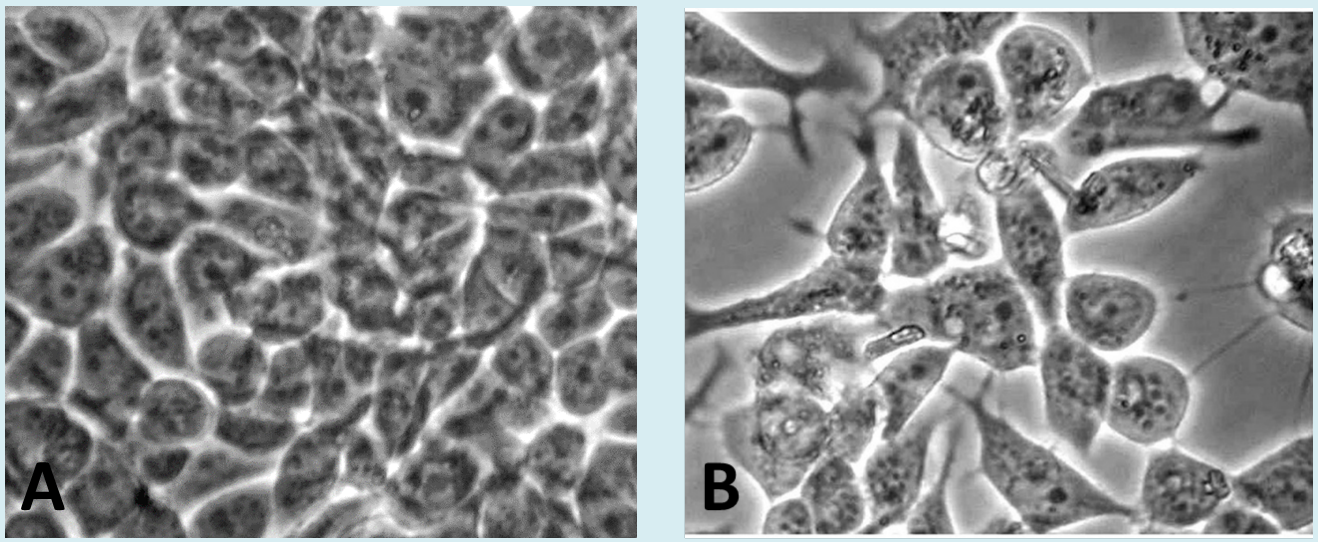


Figure 1: Phase-contrast photomicrograph of MCF-7 cells incubated in medium containing 0.01% DMSO as vehicle control (A) or in medium with the vehicle containing 10^{-8}M tamoxifen - TAM (B). Microscopic examination revealed an alteration in shape and prominent cytoplasmic extensions in MCF-7 cells when treated with tamoxifen for 48 hours (A- Vehicle control versus B- 10^{-8}M TAM).

Effects of Tamoxifen on Cell Viability (A) And Relative Levels of Galectin-3 (B) and Laminin (C)

Table 1 (A) represents results from the cell viability assay of MCF-7 cells exposed to tamoxifen for 48 h. A significant and dose-dependent decrease in cell viability was observed with

10^{-6}M (~50% reduction) or 10^{-8}M TAM (~20% reduction) (*P < 0.05*) compared to the DMSO control. The ELISA data from a single 48-hour exposure in **Table 1** (B) revealed that TAM at 10^{-8} M and 10^{-6} M concentrations decreased the relative galectin-3 levels (*P < 0.05*). Tamoxifen did not reduce laminin levels (C), but as expected, E_2 moderately increased (~15% increase) laminin.

Treatment	Percent viable cells (A)	Percent gal^{-3} (B)	Percent LN (C)
Media	96.2 ± 4.3	100	100
0.01% DMSO	98.7 ± 4.7	90.6 ± 5.8	100
10^{-6} M E2	98.8 ± 4.8	110.0 ± 5.7	116 ± 6.5
10^{-8} M E2	96.5 ± 4.2	90.3 ± 4.6	114 ± 5.2
10^{-6} M TAM	52.5 ± 5.4*	54.7 ± 3.2*	95.4 ± 5.8
10^{-8} M TAM	76.9 ± 6.1*	59.5 ± 2.0*	99.0 ± 6.5

Table 1: Effects of a single 48-hour exposure on the viability of cultured MCF-7 cells (A) and relative galectin-3 (gal-3, B) and laminin (LN, C) levels as compared to the Media control.

Data represent the mean and SEM from 3 separate experiments. * denotes a statistical and dose-dependent decrease ($p<0.05$) in cell viability (A) with both 10-6M and 10-8M tamoxifen (TAM) when compared to the Media, DMSO controls, or both 17β-estradiol (E2) exposures. The relative galectin-3 (gal-3) levels (B) were decreased ($p<0.05$) by both 10-6M and 10-8M tamoxifen (TAM) exposures (asterisks), whereas the relative laminin levels (C) did not change significantly.

Effect of Tamoxifen on the Cell Cycle

Flow cytometric analysis of the cell cycle data, showing a single 48-hour exposure, is presented in Table 2. TAM at 10^{-6}M significantly (*P < 0.05*) reduced DNA in all phases, including the S phase, of MCF-7 cells compared to the DMSO control. TAM at 10^{-8}M significantly decreased (*P < 0.05*) G2/M phase.

Treatment	Phases Of Cell Cycle		
	G_0/G_1	S	G_2/M
Media	67.3+2.4	11.7+0.7	19.7+1.1
0.01% DMSO	63.6+1.5	14.5 +1.5	20.3+1.4
10^{-6} M E2	76.7+4.7	15.7 + 1.7	20.2+1.6
10^{-8} M E2	75.5+4.8	16.4+0.6	17.1+1.4
10^{-6} M TAM	18.8 + 1.0*	6.1+0.6*	9.8+0.3*
10^{-8} M TAM	59.7+2.2	10.7+0.6	11.1+1.3*

Table 2: Effects of a single 48-hour exposure on flow-cytometric cell cycle analysis of cultured MCF-7 cells.

Data represent the mean and SEM from 3 separate experiments. * denotes statistical significance ($p<0.05$) compared to Media, DMSO controls, or both E2 exposures.

Immunofluorescence Microscopy

The cellular localization of galectin-3 was established by immunostaining both control and experimental (E_2 and TAM-exposed) cells using anti-galectin-3 antibodies (Figure 2). Cytoplasmic and nuclear localization of galectin-3 was evident in the control groups. With E_2 10^{-6}M and 10^{-8}M exposures, the staining was highly visualized both on the surface and in the nucleus of MCF-7 cells.

However, in 48-hour treatment with TAM 10^{-6}M, the staining of galectin-3 was greatly diminished. Control labeling of the cells with only secondary antibodies was negative (data not shown).

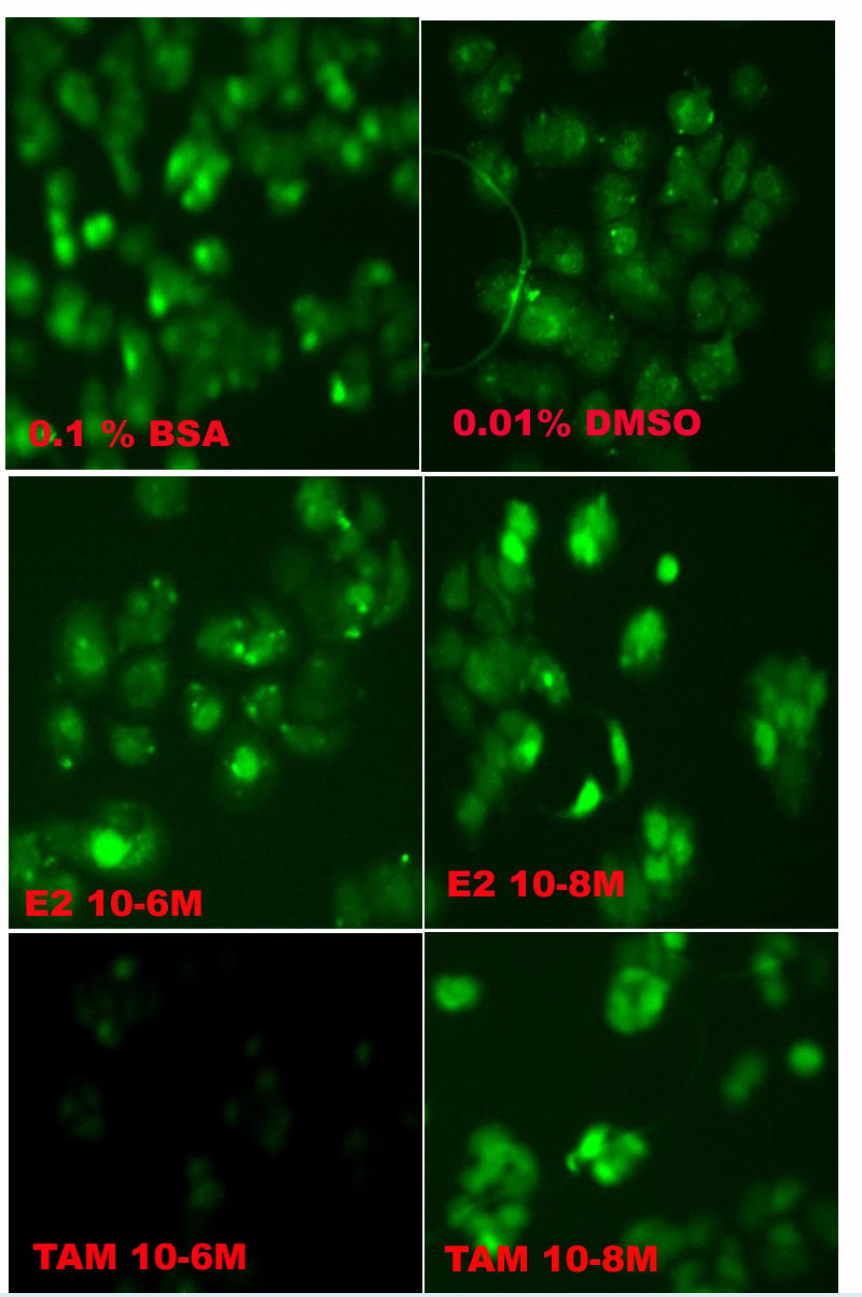


Figure 2: Photomicrograph of fluorescent-labeled galectin-3 localization in cultured MCF-7 cells. MCF-7 cells were cultured on coverslips and incubated for an additional 48 hours in a medium containing various exposures as described in the Materials and Methods. The cells treated with 10^{-6}M tamoxifen (10^{-6}M TAM) exhibited a shorter size with decreased galectin-3 labeling.

Galectin-3 and Laminin Immunolocalization (Western Blot)

The expression levels of galectin-3 or ECM protein laminin were determined by Western blot analysis (Figure 3). We used β-actin as a housekeeping protein for equal loading and transfer. The immunoblot analysis revealed no relative change in galectin-3 expression in the 24-hour treatment group (Figure 3A). However, in the 48-hour treatment group, the relative expression of the galectin-3 protein was significantly decreased with 10^{-6}M TAM (Figure 3B). We have also observed a decrease in galectin-3 with 10^{-6}M TAM in the 72-hour treatment group (Figure 3C). In comparison, the relative expression of laminin in the 48 h treatment group remained unaltered with 10^{-6}M TAM (Figure 3D). However, we have observed an increase in laminin with both 10^{-6}M and 10^{-8}M E_2.

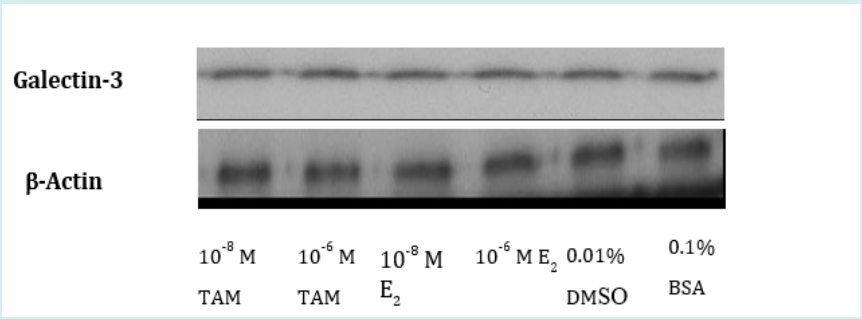


Figure 3A: Western blot of galectin-3 in MCF-7 Cells - 24 hour Treatment.

Quantification of Western blot data in terms of ratio (galectin-3/β-actin)

Exposure	0.1% BSA	0.01% DMSO	10-6M E2	10-8M E2	10-6MTAM	10-8M TAM
24h	0.40 ± 0.002	0.40±0.001	0.35±0.0001	0.38±0.003	0.42±0.008	0.40±0.0004

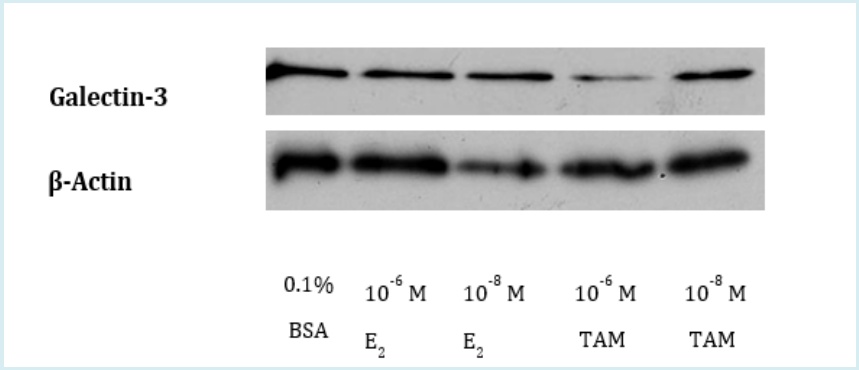


Figure 3B: Western blot of galectin-3 in MCF-7 Cells - 48 hour Treatment.

Quantification of Western blot data in terms of ratio (galectin-3/β-actin).

Exposure	0.1% BSA	0.01% DMSO	10^{-6}M E2	10^{-8}M E2	10^{-6}MTAM	10^{-8}M TAM
48h	0.50 ± 0.002	ND	0.65±0.0001	0.60±0.003	0.15±0.003	0.35±0.0002

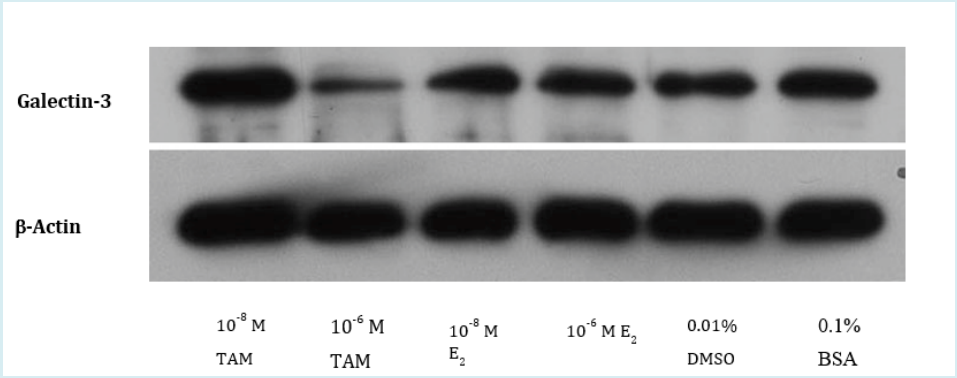


Figure: 3C: Western blot of galectin-3 in MCF-7 Cells - 72 hours Treatment.

Quantification of Western blot data in terms of ratio (galectin-3/β-actin).

Exposure	0.1% BSA	**0.01% DMSO**	**10^{-6}M E2**	**10^{-8}M E2**	**10^{-6}MTAM**	**10^{-8}M TAM**
72h	0.40 ± 0.001	0.35±0.002	0.40±0.002	0.35±0.002	0.15±0.003	0.45±0.004

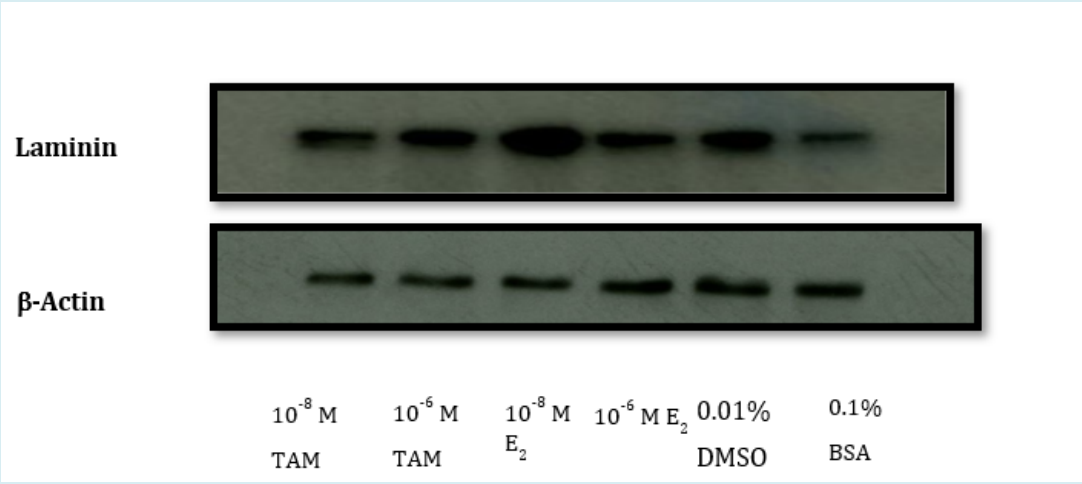


Figure 3D: Western blot of laminin in MCF-7 Cells - 48 hour Treatment. Quantification of Western blot data in terms of ratio (laminin/β-actin)

Exposure	**0.1% BSA**	**0.01% DMSO**	**10^{-6}M E2**	**10^{-8}M E2**	**10^{-6}MTAM**	**10^{-8}M TAM**
48h	0.50 ± 0.002	0.60±0.001	0.80±0.0001	1.0±0.003	0.60±0.008	0.50±0.0004

Discussion

For over three decades, Tamoxifen has been widely accepted as an adjuvant endocrine drug. It can bind to ERα and act as an antagonist through competition with estradiol. It modulates gene expression in ERα-positive breast cancer cells [17,18]. In the present study, we demonstrated dose-dependent morphological changes in MCF-7 cells exposed to tamoxifen. Earlier, we reported that relatively higher TAM concentrations (10^{-4}M) induced morphological changes and reduced DNA in MCF-7 cells when exposed for 24 hours [19]. Others observed morphological changes in apoptosis or PCD, which is cell condensation and the reorganization of the cell cytoskeleton [20]. We also noted earlier a reduced level of F-action expression in MCF-7 cells with 10^{-4}M of TAM exposure in 24 hours [19]. Cell preparation and execution of apoptosis are associated with depolymerization and cleavage of actin and other cytoskeletal proteins [21-30]. However, the role of galectin-3 and its association with the ECM protein laminin was not reported in earlier studies.

Western blot analysis was utilized to determine the effect of tamoxifen on galectin-3 and laminin expressions in MCF-7 breast cancer cells. Over the 24-hour incubation period, no significant changes were observed. However, in 48- and 72-hour periods, TAM (10^{-6}M) treatment decreased galectin-3 expression in MCF-7 cells compared to the controls, and laminin levels remained unaffected. Even with the morphological changes due to the exposure of MCF-7 cells to tamoxifen, the ECM protein laminin didn't show any alterations. Still, the cellular protein galectin-3 demonstrated marked reductions beyond the 24-hour exposure period. Laminin, a key extracellular matrix component, plays a critical role in maintaining cellular adhesion, polarity, and cell-matrix interactions. The preservation of laminin expression suggests that tamoxifen may not broadly disrupt cell-ECM interactions, underscoring a nuanced mechanism through which tamoxifen rather selectively modulates intracellular functions. Interestingly, this finding raises questions about the specificity of tamoxifen and how it modulates cell functions without compromising the architecture of the ECM, affecting the structural and signaling components.

Here, we see a differential relationship between tamoxifen [31] and estrogens [32-36] regarding the inhibitory effects on cell proliferation of MCF-7 growth. Therefore, the downregulation of galectin-3 may be instrumental in cell growth inhibition or even cell death caused by tamoxifen. Earlier, at relatively higher concentrations, tamoxifen-induced apoptosis in MCF-7 cells was reported by our group [37].

We have demonstrated here that tamoxifen decreases in all phases, including the S-phase, resulting in reductions in DNA synthesis in MCF-7 cells. Tamoxifen has been known to be an inducer of apoptosis in a dose-dependent manner [38]. It is also known to appear anti-proliferative in its induction of MCF-7 growth at higher concentrations of administration [39]. In this study, tamoxifen exposure for extended periods was associated with an apparent inhibition of galectin-3 protein in cells without affecting the ECM component, such as laminin. This is the first report to demonstrate an association of galectin-3 without the involvement of laminin with the transformed phenotype of MCF-7 cells due to tamoxifen treatment. It was reported that galectin-3 was a differentially expressed and downregulated gene in LCC9 cells. However, it was normalized with the therapy of Valproic acid and hydralazine. They also found animal models using in-utero ethinyl estradiol tumors in rats. They predicted that these genes could be the reason for poor survival in TAM-treated ER-positive breast cancer patients [40].

In BT-549 mammary epithelial cells, the introduction of recombinant galectin-3 resulted in tumorigenic properties [41]. This research group later reported [42] that the downregulation of galectin-3 suppressed the tumorigenicity of highly malignant triple-negative MDA-MB-231 breast cancer cells.

In summary, tamoxifen exhibited cytostatic effects on MCF-7 breast cancer cells. Morphological analysis revealed that TAM caused MCF-7 cell shrinkage and cytoplasmic extension, suggesting a loss of epithelial integrity. Correspondingly, cell viability was significantly reduced. Tamoxifen exposure also significantly reduced S-phase DNA content, indicating cell cycle arrest and reduced proliferative capacity. Notably, galectin-3 protein levels were decreased considerably after longer TAM treatment, while laminin expression remained unchanged, implying selective modulation of intracellular signaling and extracellular matrix components. These results suggest that TAM impairs breast cancer cell survival by altering morphology, inhibiting proliferation, and downregulating galectin-3, a multifunctional protein implicated in pathophysiology.

Acknowledgement

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Conflicts of Interest

The authors declare no conflict of interest.

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