# Effect of brefeldin A on membrane localization of MUC1 mucin and adhesive properties of cancer cells

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Transmembrane glycoproteins play a significant role in cancer cells adhesion and metastatic process, just for that reason the glycosylation inhibitors are used to change the glycan structure and in this way the membrane expression of glycoproteins. The inhibitory effect of brefeldin A (BFA) on the expression of some glycoproteins: MUC1 mucin and $\alpha 2\beta 1$ integrin on cell surface of breast (MCF-7 and MDA-MB-231 lines) and endometrial (Ishikawa line) cancer cells was evaluated in our study. In MCF-7 and MDA-MB-231 cells, a decrease in MUC1 expression depended on brefeldin A concentration and equaled about 40% in cells treated with 1mg% of drug. In Ishikawa cells, a decrease in MUC1 expression was lower and amounted to about 25%. The expression of $\alpha 2\beta 1$ integrin was greatly inhibited in brefeldin-treated MCF-7 and Ishikawa cells, though it was unchanged in MDA-MB-231 cells.

A decrease in MUC1 mucin and $\alpha 2\beta 1$ integrin level reduced the adhesive properties of BFA-treated cells. Adhesion to type I collagen was greatly diminished in BFA-treated MCF-7 and Ishikawa cells (above 70%), and to a lesser degree in MDA-MB-231 cells (about 50%); which was mainly caused by the inhibited integrin expression. These findings have proved that brefeldin A, by changing the surface glycoproteins level, can alter carcinoma cells adhesion to extracellular matrix proteins.

*Key words: MUC1, integrins, cancer cell lines, brefeldin A, cell adhesion*

Approximately 80% of secreted and cell surface proteins are glycosylated. The glycosylation changes associated with cancer include the under- and/or overexpression of naturally present glycans. MUC1 is a one of many membrane glycoproteins of which level changes with malignant transformation. This heavily O-glycosylated protein is normally expressed at the apical borders of glandular epithelial cells. Following malignant transformation, MUC1 often becomes highly overexpressed, loses its apical restriction and displays aberrant glycosylation. The functions attributed to MUC1 include those generally associated with mucins, but additionally, MUC1 is considered to be an effective inhibitor of both cell-cell and cell- extracellular matrix interactions. The increased expression and aberrant glycosylation of this mucin have been implicated in increasing metastatic potential [1–3].

Glycans play a variety of roles in the behavior of cancer cells [4], therefore the treatment with glycosylation inhibitors is used to study the functional role of cell surface glycoproteins in cancer. Tunicamycin is frequently used to inhibit N-glycosylation [5, 6], whereas benzyl-N-acetyl-α-galactosaminide to constrain O-glycans elongation, and they both consequently disrupt apical targeting of some membrane glycoproteins [7, 8]. Brefeldin A (BFA) is known as an inhibitor of glycoprotein secretion, which blocks the transport pathway from the endoplasmic reticulum (ER) to the Golgi complex [9, 10]. Additionally, it has been shown that this drug collapses the Golgi complex into the ER, redistributes processing enzymes normally resident in the Golgi to the ER, and uncouples the proximal and distal regions of the secretory pathway [11–13].

In our previous studies, the influence of tunicamycin and benzyl-α-GalNAc on properties of MUC1 glycoprotein was evaluated in cancer cells [14, 15]. At present, an attempt has been made to estimate the effect of brefeldin A on the membrane expression of some glycoproteins: MUC1 mucin and integrins in different cancer cell lines of the epithelial origin. Taking into consideration the relation between the membrane

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protein expression and the adhesive properties of these cells, the changes in adhesion to collagen of BFA-treated cells and the participation of MUC1 and α2β1 glycoproteins in this process were also examined in our present study.

## Materials and Methods

*Materials.* Brefeldin A (BFA), collagen I, SDS, protease inhibitors cocktail, ABTS liquid substrate system, trypan blue and culture media were purchased from Sigma Chemicals; monoclonal anti-MUC1 antibody (MAb 4058) was from Chemicon International; monoclonal anti-MUC1 antibody conjugated with fluorescein isotiocyanate (HMPV-FITC) and $IgG_1$-FITC isotype control were purchased from BD Pharmingen; monoclonal anti-integrin α2β1 antibody (P1E6) was from Dako; culture flasks (25 and 75 $cm^2$) were from Sarstedt (USA); Labtek chamber slides 4-well, microtitre plates (6-well and 96-well) were from Nunc (Denmark); EC10 inhibitor of α2β1 integrin was kindly provided by Dr. C. Marcinkiewicz (Temple University, PA, USA). All other chemicals were of analytical grade purity from commercial sources.

*Cell culture.* Breast cancer cell lines (MCF-7, MDA-MB-231) and endometrial carcinoma cells (Ishikawa) were purchased from the American Type Culture Collection (Rockwille, MD). Ishikawa and MCF-7 cell lines were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin at 37°C in 5% $CO_2$ in air. MDA-MB-231 cell culture was maintained in Leibowitz L-15 medium with 0.2 mM glutamine and 10% FBS at 37°C in humidified atmosphere of air. Subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium free phosphate buffered saline, counted in hemocytometers and inoculated in 96-well plates (for cell ELISA test), in six-well plates (for flow cytometry) or in chamber slides (for immunohistochemistry). After reaching 80% of confluence cells were treated with brefeldin A at a concentration of 0.2, 0.5 or 1.0 mg% for 24 h. Control cells were inhibitor free. Brefeldin A was dissolved in ethanol, then diluted with culture medium to a final concentration of ethanol ≤ 0.01%, which had no influence on cell viability. In all experiments, >95% of cells showed viability as assessed by trypan blue assay. Two individual cultures were grown simultaneously for controls and inhibitor-treated cells in three independent experiments.

*Cancer cells ELISA.* MUC1 expression was evaluated in ELISA test according to Strindhall et al. [16] with some modifications. Briefly, the control and BFA-treated cells were washed once with PBS and fixed with 0.1% formaldehyde/PBS for 10 min at room temperature, then endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min. After two washes with PBS the cells were incubated with PBS/5% BSA for 1h at room temperature or overnight at 4°C to block nonspecific binding sites. The cells were washed once with PBS/0.05% Tween 20 and once with PBS and then incubated with anti-MUC1 primary antibody (MAb 4058) at a 1:500 dilution in PBS/1% BSA for 2 h at 37°C with gentle rocking. Cells were washed twice with PBS and once with PBS/0.05% Tween 20 and incubated with secondary antibody conjugated with horseradish peroxidase at a dilution of 1:1000 in PBS/1% BSA for 2h at room temperature with gentle rocking, then four times washed with PBS and color reaction was performed by using ABTS reagent. Absorbance was measured at 405 nm.

*Flow cytometry.* Cells were detached from tissue culture plates with 0.2% EDTA in PBS, pH 7.4 and washed in PBS containing 1% BSA. To determine MUC1 expression on the surface of control and BFA-treated (1 mg%) cells, the detached cells ($1\times10^5$ – $1\times10^6$) were incubated with fluorescein isotiocyanate (FITC)-conjugated mouse anti-human MUC1 monoclonal antibody in the dark at 4°C for 30 min. Mouse $IgG_1$-FITC antibody was used as a negative control. All studied cells were analyzed for fluorescence intensity by flow cytometry (Coulter Epics XL).

*Immunohistochemistry.* Cells grown on chamber slides were fixed in Cytofix, and then washed twice in PBS buffer, pH 7.4. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 5 min. After washing with Tris/HCl buffer solution, pH 7.4, slides were incubated with primary antibodies anti-MUC1 (MAb 4058) or with anti-α2β1 integrin (P1E6). For detection the Dako Cytomation LSAB+ System HRP kit was used according to the instructions of the manufacturer, employing the streptavidin-biotin technique. All sections of slides were examined independently by two investigators under a standard light microscope using ×200 and ×400 magnification.

*Cell adhesion assay.* A cell adhesion assay was based on the method described by Dufour et al. [17], with some our modifications [14]. Adhesion assay was performed with control and brefeldin-treated cells. Cells for adhesion assay were suspended in culture medium containing 0.3% BSA and reseeded at a density of $5 \times 10^4$ cells/well on collagen type I. Cells were allowed to adhere for 60 min at 37°C. Non-adherent cells were removed by washing 3 times with PBS. Adherent cells were fixed with 96% ethanol for 10 min, then washed and stained with 0.1 % crystal violet for 30 min. Stained cells were then washed in tap water, air dried and lyzed overnight in 0.1% Triton X-100. The absorbance at 620 nm, determined by means of ELISA-reader (Sunrise, Tecan), was proportional to cell number. Each data point was calculated from three separate experiments performed in quadruplicate and expressed as the mean ± standard deviation (SD). Nonspecific cell adhesion, measured on BSA-coated wells, was subtracted.

*Assay of α2β1 integrin influence on cell adhesion.* To test the participation of α2β1 integrin in cell adhesion to type I collagen, a part of cell suspension was preincubated with EC10 (2 mg/0.1 ml) for 30 min at 37°C, and added to each collagen-coated well ($5 \times 10^4$ cells/well) in quadruplicate. This compound is an analogue of EMS16 and a potent and selective *in vitro* inhibitor of α2β1 integrin [18]. A further

procedure was the same as described above for the adhesion test. Results were expressed as a percentage of optical density (OD) value of cells preincubated with EC10 in comparison to OD of control cells (without BFA and without EC10 preincubation).

*Assay of MUC1 influence on cell adhesion.* To test the effect of MUC1 on cancer cell adhesion to collagen type I, a part of cell suspension was preincubated with anti-MUC1 MAb 4058 (1:25 diluted) for 30 min at 37°C. A further procedure was the same as described above for the adhesion test. Results were expressed as a percentage of OD value of cells with antibody in comparison to OD of control cells (without BFA and without antibody).

*Statistical analysis.* Data were presented as means ± SD. Statistical analysis was performed using Student's *t*-test to determine the significance of differences between means. A $p<0.05$ was considered as statistically significant.

## Results

*Effect of brefeldin on membrane localization of MUC1 mucin and integrins in cancer cells.* The breast (MCF-7, MDA-MB-231) and endometrial (Ishikawa) cancer cells were cultured with BFA at a concentration of 0.2-1 mg% for 24h, then the level of glycoproteins (MUC1 and α2β1 integrin) on the cell surface was determined by ELISA, flow cytometry and immunohistochemical methods.

As detected in ELISA test (Fig.1), the MUC1 expression was inhibited in all cell lines, with the greatest degree in MDA-MB-231. This inhibition depended on brefeldin A concentration in breast cancer cells and, in regard to non-treated cells, it ranged from 14% to 41% in MCF-7, and from 27% to 43% in MDA-MB-231. In endometrial cancer cells (Ishikawa line), the effect of brefeldin A was lower and reached 24% of inhibition.

These results were confirmed by flow cytometry method (Fig.2). In MCF-7 and MDA-MB-231 cells, about 40% of inhibition of MUC1 expression was found after treatment with 1mg% of BFA. Some differences were observed in Ishikawa cells, in which about 50% of a decrease in MUC1 expression was detected in flow cytometry and only 23% in ELISA method.

Immunohistochemical examination of the effect of brefeldin A on the exposure of transmembrane glycoproteins also demonstrated a considerable decrease in MUC1 expression on MCF-7 and Ishikawa cells, about 30% and 40%, respectively (Tab.IA,C); in contrast, MUC1 exposure was nearly unchanged with the increased concentration of BFA on MDA-MB-231 cells (Tab.IC). In this study, we also examined the influence of brefeldin A on the expression of α2β1 integrin, present as collagen type I receptor on these cells. The marked inhibition of this integrin expression was demonstrated in MCF-7 and Ishikawa cells, similarly to mucin (Tab.IA,B). However, no inhibition of the integrin expression was observed in MDA-MB-231 cells (Tab.IB).

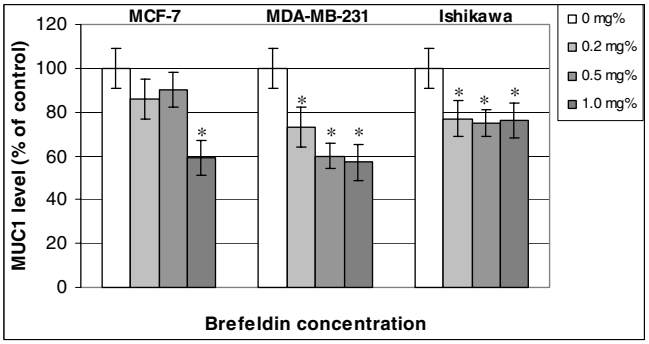


**Figure 1. The effect of brefeldin A on MUC1 protein expression on the cell surface of breast and endometrial cancer cells monitored by cell ELISA. Cells were treated with brefeldin A at a concentration: 0.2; 0.5; 1 mg% for 24 h and MUC1 level in control non-treated cells was assumed as 100%. Data are shown as means ± SD of two independent experiments. *A difference between treated and control cells was statistically significant.**

**Table I. The effect of brefeldin A on the membrane glycoproteins expression, detected by immunohistochemical method in cancer cells[a]. A – MCF-7, B – MDA-MB-231, C- Ishikawa.**

A. MCF-7

| Brefeldin A concentration [mg%] | Detection of MUC1 | Detection of α2β1 integrin |
|---|---|---|
| 0 | +++/++ (83%) | + (26%) |
| 0.2 | +++ (80%) | + (15%) |
| 0.5 | ++ (60%) | +/++ (10%) |
| 1.0 | ++ (55%) | +/++ (8%) |

B. MDA-MB-231

| Brefeldin A concentration [mg%] | Detection of MUC1 | Detection of α2β1 integrin |
|---|---|---|
| 0 | ++ (60%) | + (15%) |
| 0.2 | ++ (60%) | + (10%) |
| 0.5 | ++ (70%) | + (10%) |
| 1.0 | ++ (75%) | ++ (15%) |

C. Ishikawa

| Brefeldin A concentration [mg%] | Detection of MUC1 | Detection of α2β1 integrin |
|---|---|---|
| 0 | +++ (85%) | +/- (33%) |
| 0.2 | +/++ (45%) | + (5%) |
| 0.5 | ++ (50%) | +/- (5%) |
| 1.0 | +/++ (45%) | +/- (<5%) |

[a]Optical intensity (scale: +, low; ++, moderate; +++, high; +/-, less than low) and percentage of these cells with positive immunostaining antigens in control and BFA-treated (24 h) cells.

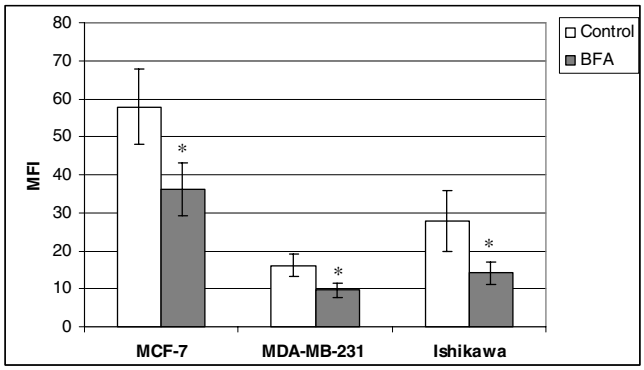


**Figure 2. Flow cytometry analysis of MUC1 protein expression on the surface of cancer cells. Average values of mean fluorescence intensity (MFI) in 3 independent experiments are shown for control and BFA-treated cells (1mg%, for 24 h). *A difference between treated and control cells was statistically significant.**

*Influence of brefeldin A on cell adhesion to collagen type I.* In order to examine the influence of BFA on cell adhesion to type I collagen, non- and BFA-treated cells (1 mg%, for 24h) were immediately used to adhesion test. The significant decrease was observed in the adhesion after brefeldin A treatment in all studied cell lines. In regard to non-treated cells, the greatest inhibition was found in MCF-7 and Ishikawa cells (73%) (Fig.3A,C – open bars); in MDA-MD-231 cells the inhibition was lower and equaled 48% (Fig.3B-open bars).

Since the presence of $\alpha 2\beta 1$ integrin receptor was confirmed in these cancer cells by immunohistochemical method (Tab.I), the participation of this integrin in adhesion to type I collagen was then tested in our study. The adhesion levels of: 1/ control cells and control cells preincubated with EC10 ($\alpha 2\beta 1$ integrin inhibitor); 2/ BFA-treated cells and -treated cells preincubated with EC10 were compared successively in the study. A considerable decrease in the adhesion level was observed in MCF-7 and Ishikawa control cells (about 70%), and insignificant in MDA-MB-231 control cells (11%), in regard to non-preincubated with EC10 cells (left side of Fig.3; open bars and hatched bars). On the contrary, EC10 blocker was non-effective in the adhesion of BFA-treated MCF-7 and Ishikawa cells, whereas the adhesion of BFA-treated MDA-MB-231 was decreased by about 60% after incubation with EC10 (right side of Fig.3; open bars and hatched bars).

Additionally, control and BFA-treated cells were preincubated with anti-MUC1 core protein antibody, MAb 4058. A small increase in the adhesion of preincubated control cells (MCF-7 and Ishikawa; 19% and 15 %, respectively) and unchanged adhesion in MDA-MB-231 control cells was found in this study (left side of Fig.3A,C and B; open bars and dotted bars). On the other hand, anti-MUC1 antibody did not change the cell adhesion in all BFA-treated cell lines (right side of Fig.3A,B,C; open-bars and dotted bars).

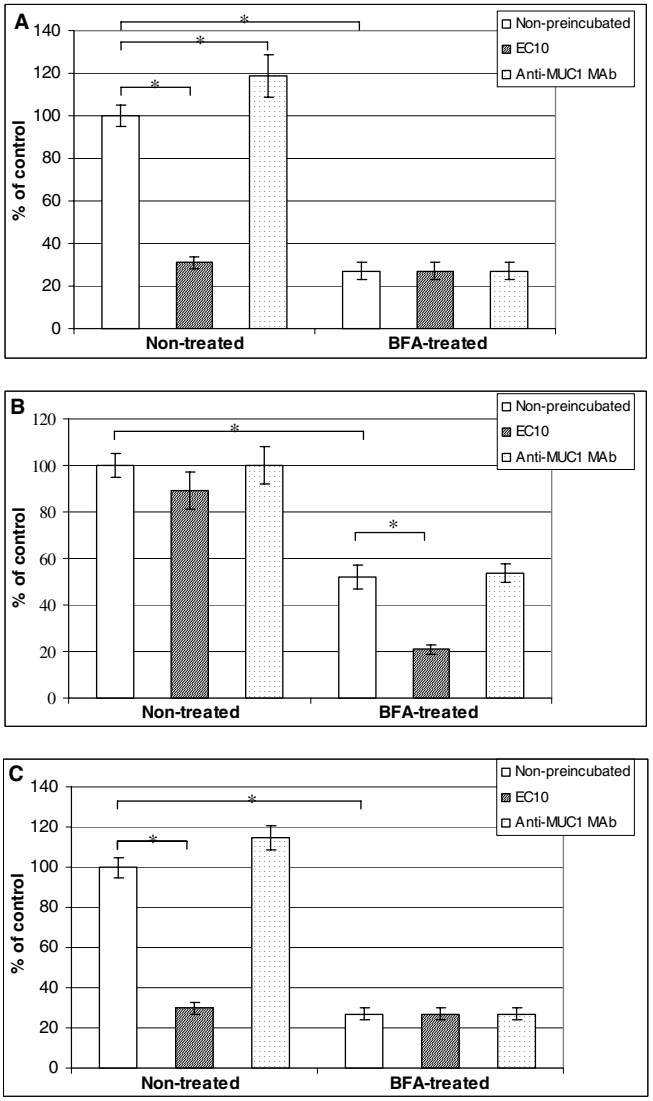


**Figure 3. Effect of brefeldin A on cancer cells adhesion to collagen type I: A – MCF-7, B - MDA-MB-231, C – Ishikawa. The cells were cultured for 24 h in standard medium with 1 mg% BFA or without inhibitor. Before the adhesion test, a part of control and inhibitor-treated cells ($2 \times 10^5$ cells) of each line, were preincubated with $\alpha 2\beta 1$ integrin blocker (EC10), or anti-MUC1 antibody. Next, the cells were added to collagen-coated wells ($5 \times 10^4$ cells/well) in quadruplicate. Adhesion of the control cells (without drug and preincubation) was assumed as 100%; adhesion of other groups of cells was expressed as a percentage of this value. Data are shown as means ± SD of two independent experiments. *A difference between marked groups was statistically significant.**

## Discussion

A good example of glycosylated tumor antigen is epithelial transmembrane mucin MUC1, which can be overexpressed by cancer cells, membrane incorporated and shed to body fluids [19]. The core protein of the mucin is processed in the lumen of the endoplasmic reticulum (ER)

by the proteolytic cleavage of the ectodomain component from the transmembrane component [20, 21]. The two fragments form a stable but noncovalent, heterodimeric complex, of which an extracellular fragment is heavily O-glycosylated during transit by the Golgi complex. After exposure on the plasma membrane, MUC1 is internalized via endocytosis and further sialylated before it is recycled to the cell surface [22]. A portion of the membrane-associated MUC1 can be shed, presumably by the second proteolytic cleavage of the ectodomain. In this way, MUC1 enters into body fluids or layers on epithelial surfaces and it can modulate the cell adhesion to ECM, contributing to the growth and metastatic properties of tumor. However, its influence varies among different tumor types and its precise function is not completely known [19].

In our earlier studies, the effects of main N- and O-glycosylation inhibitors on the expression of some membrane proteins (MUC1 mucin and $\alpha 2\beta 1$ integrin) were examined in human endometrial and breast cancer cell lines (Ishikawa and MCF-7). Those experiments showed that applied glycosylation inhibitors altered the glycosylation pattern of cell surface glycoproteins sufficiently enough to alter the adhesion of cancer cells to ECM proteins [14, 15].

Searching for another way to influence the exposition of adhesion and anti-adhesion molecules on the cell surface, we studied recently the effect of brefeldin A on breast and endometrial cancer cells. Our results obtained in flow cytometry and ELISA methods, indicated the highest MUC1 expression on the surface of MCF-7 cells, and lower in MDA-MB-231 and Ishikawa, which was inhibited in all cell lines after BFA-treatment. A similar concentration-dependent, inhibitory effect of BFA was observed in both breast cancer cell lines, whereas it was lower in Ishikawa cells. Nevertheless, in immunohistochemical staining, the maximum decrease in MUC1 expression was showed on the surface of BFA-treated Ishikawa cells, although only approximate results can be obtained with this technique. Several other authors have also demonstrated that BFA blocks the membrane transport of some proteins, such as class I glycoproteins [23] and toxins [24, 25], and inhibits glycosaminoglycan elongation and sulfation reactions [26].

It is well known that MUC1 mucin as transmembrane glycoprotein can modulate the cell adhesion (27–29). In our studies the increased adhesion of cells with greater MUC1 expression (MCF-7 and Ishikawa), and unchanged adhesion of MDA-MB-231 cells were observed after incubation with anti-MUC1 antibody. This antibody reduces a steric hindrance caused by overexpression of MUC1 and probably therefore does not influence the cells with a lower MUC1 level. This was confirmed by the fact that after preincubation with anti-MUC1 antibody, the adhesion of BFA-treated cells did not change, which may be caused by the low expression of MUC1 after the action of brefeldin.

The brefeldin effect on cell adhesion to collagen was significant and depended on the changes in the expression of membrane glycoproteins, such as MUC1 and integrins. The adhesion was more diminished in BFA-treated MCF-7 and Ishikawa cells than in MDA-MB-231. In our earlier studies we demonstrated that the increased adhesion is caused by the reduction in MUC1 expression [14, 15]. Thus, the results presented above were surprising. However, an inhibitory effect of BFA on adhesion may have resulted from a total changes in MUC1, integrins and other membrane glycoproteins expression.

The integrins belong to the cell surface adhesion receptors. They are the family of membrane glycoproteins that mediate adhesion to either components of extracellular matrix or to other cells [30]. The main function of $\alpha 2\beta 1$ integrin in the adhesion of MCF-7 and Ishikawa cells to type I collagen was confirmed by an inhibitory effect of EC10 blocker on the attachment of control cells (about 70% of inhibition). On the contrary, the adhesion was only slightly diminished by EC10 blocker (about 10% of inhibition) in MDA-MB-231 cells. It may be caused by the participation of other integrins in these cells adhesion, which is in accordance with previous findings on the role of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ collagen receptors in the attachment of MDA-MB cells to collagen type I [31].

We proved that BFA inhibits, but not blocks totally the membrane exposition of some glycoproteins: MUC1 mucin and $\alpha 2\beta 1$ integrins, and by this way diminishes the cell adhesion to ECM proteins. A fundamental role in the synthesis and processing of glycoconjugate oligosaccharides, as well as in the sorting of glycoconjugates to different cell compartments plays the Golgi complex [32]. Although newly synthesized proteins appeared to be retained in the ER of BFA-treated cells, this drug does not generally inhibit posttranslational processes. Since BFA mixes up compartments, certain proteins normally resident in the ER can be inappropriately processed by the enzymes that normally act only in the Golgi complex [11, 33].

Glycosylation touches many diverse components of the cell, but a small number of glycosylation inhibitors have been already found as potential drugs [34–36]. The research has been done to find inhibitors that have a selective influence on some adhesion molecules important in the cell-cell and cell-extracellular matrix interactions. The results of our studies demonstrated a decrease in MUC1 mucin and $\alpha 2\beta 1$ integrin expression on the cellular membrane after BFA treatment. The BFA influence on adhesion seemed to be caused to a greater extent by the changes in integrin than MUC1 expression in all the study cancer cell lines.

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