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ORIGINAL RESEARCH

Effect of *O*-Glycosylation and Sialylation Inhibitors on Classical NLS-Dependent Nuclear Protein Import in HT29-MTX Human Colon Cancer Cells

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Abstract: Glycosylation, including *O*-linked, mucin type, has an import role in the function and activity of many proteins, particularly those that are secreted or transcellular. Previous studies have indicated that mucin-type *O*-linked glycans may also be carried by intracellular proteins such as the stress-related protein Orp150 that is involved in classical nuclear localization sequence (NLS)-dependent nuclear protein import. This study investigated the influence on NLS-dependent nuclear protein import of the potent *O*-glycosylation inhibitor, Benzyl-GalNAc; the sialylation inhibitor, 5'CDP; the Golgi proton pump inhibitor, bafilomycin; and the pro-inflammatory cytokine, TNFα. Treatment of the mucus-secreting human colon cancer HT29-MTX cells with each of these agents caused a global increase of the cellular core 1 carbohydrate structure (galactose β1,3 N-acetylgalactosamine), one of the precursor structures of the complex mucin-type glycans. Benzyl-GalNAc treatment also increased the expression of the core 1 structure on cell surface glycoconjugates and caused a generalised decrease in sialylation. Hsp70 nuclear translocation upon heat stress, a process that is mediated by the classical NLS pathway, was, however, not affected by pre-treatment of the cells with any of these agents. This suggests that *O*-linked and/or sialylated glycans are unlikely to be involved in the classical NLS-dependent protein import mechanism.

Keywords: *O*-glycosylation, nuclear import, sialic acid, TF antigen

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Introduction

Glycosylation of cellular proteins plays a very important regulatory role in determining their activity and function.[1–4] One common glyco-modification is the attachment of *O*-linked mucin-type glycans.[5] This occurs as a multi-stepped post-translational process initiated by the addition of *N*-acetyl-galactosamine (GalNAcα-) to a serine or threonine residue of the fully folded/assembled protein. The formation of GalNAcα-Ser/Thr structure (Tn-antigen) can be further modified by the addition of either a sialic acid residue (sialyl Tn), or a *N*-acetyl-glucosamine (GlcNAc) residue to form GlcNAcβ1,3GalNAcα- (core 3) or a galactose residue to form the Galβ1,3GalNAcα- (core 1) structure. The core 3 and 1structures are then further extended to form long and branched complex *O*-glycans. The core 1 Galβ1,3GalNAcα- carbohydrate structure, also known as the Thomsen-Friedenreich (TF or T) antigen, is therefore one of the earlier precursor structures of the extended mucin-type glycans. It behaves as an oncofetal antigen, with increased expression in cancerous and precancerous conditions,[6] partly as a consequence of disarray of the Golgi apparatus.[7]

Our previous studies have shown that interactions between TF-antigen expressed on the surface of cancer cells and anti-TF antibody[8] or the TF-binding lectin, peanut agglutinin (PNA), stimulates colonic epithelial proliferation *in vitro* in cancer cells[9] and *in vivo* in individuals who eat peanuts.[10] The proliferative effect of the PNA-TF interaction is linked to phosphorylation of the hepatocyte growth factor receptor c-Met and subsequent activation of the ERK cell growth signaling.[11] On the other hand, interaction of cell surface TF antigen with two other TF-binding lectins from the common edible mushroom *Agaricus bisporus* (ABL) and jackfruit (jacalin) in both cases produced the opposite effect and induced reversible inhibition of cell proliferation.[12,13] The jacalin-mediated inhibition of cell proliferation is found to be associated with tyrosine phosphorylation of the tumour suppressor PHAPI that causes dissociation of PHAPI from complex with protein phosphatase PP2A with subsequent PP2A activation and dephosphorylation of the ERK signalling proteins.[14]

The growth inhibitory effect of ABL, unlike that of jacalin, requires internalisation of the lectin[15] and its interaction with an N-terminally truncated cytoplasmic form of the stress-related protein Orp150 (oxygen-regulated protein 150) and consequent prevention of the classical nuclear localization sequence (NLS)-dependent nuclear import.[16] We found that treatment of Orp150 with sialidase reveals binding of Orp150 by PNA, which otherwise does not bind, and further treatment of the sialidase-pre-treated Orp150 with *O*-glycanase, which specifically removes unsubstituted TF, abolishes the PNA binding. These results imply that a sialylated TF structure is carried by this truncated cytoplasmic Orp150 form. This demonstration of a mucin-type *O*-glycan structure on a cytoplasmic protein involved in nuclear protein import taken together with the accumulating evidence for the presence of complex glycans on intracellular proteins[17] led us to investigate the influence of mucin-type *O*-glycans on NLS-dependent nuclear translocation.

In this study, we assessed the effects on nuclear protein import of the *O*-glycosylation inhibitor—Benzyl-GalNAc, the sialylation inhibitor—5'CDP, and the Golgi proton pump inhibitor—bafilomycin. We also studied the effect of the pro-inflammatory cytokine, TNFα, in view of its inhibitory effect on *O*-glycosylation seen in human epithelial cells.[18,19] We used nuclear import of heat shock protein70 (Hsp70) upon heat stress, a process that is known to be mediated by the classical NLS pathway,[20] as an endogenous transport marker in mucus-secreting human colon cancer HT29 cells.

Materials and Experimental Procedures

Materials

Biotin conjugated—peanut agglutinin (PNA) and *Maackia amurensis* lectin II (MAL-II) were obtained from Vector Laboratories (Peterborough, UK). Benzyl-GalNAc, 5'CDP, Non-enzymatic Cell Dissociation Solution and anti-Hsp70 monoclonal antibody ware from Sigma (Poole; UK). TNFα was obtained from Cambridge Bioscience (Cambridge, UK).

Cells

The HT29-MTX human colon cancer cells were kindly provided by Dr. Lesuffleur (INSERM U560, Lille, France) and cultured in DMEM containing 10% fetal calf serum in standard cell culture conditions.

These cells are a homogeneous mucus-secreting subpopulation of the parental HT29 cells and were obtained by stepwise adaptation of the parental HT29 cells to 10^{-6} or 10^{-5} M methotrexate (MTX).[21]

Treatment of cells with inhibitors of *O*-Glycosylation and sialylation and assessment of total cellular expression of TF and sialic acid by slot blotting

HT29-MTX cells were cultured in 24-well plates to 60% confluence before introduction of the putative glycosylation inhibitors for 24 to 72 hr at optimal concentration of each agent that has been shown previously to effectively inhibit cellular *O*-glycosylation (100 nM Bafilomycin, 2 ng/ml TNFα, 1 mM 5'CDP and 2 mM Benzyl-GalNAc). The cells were lysed and slot blot were performed. The slots were incubated with 1% BSA/PBS for 30 min before application of biotinylated TF-binding peanut agglutinin (PNA) or sialic acid-binding *Maackia amurensis* lectin II (MAL-II) (1 µg/ml) in 1% BSA/PBS for 1 hr at room temperature. After washing, the blots were incubated with peroxidase-avidin (1:2000 dilution in a 1% BSA/PBS) for 1 hr and developed with Super Signal West Dura Extended During Substrate (Pierce) and visualised using a Fluor-S Imager (Bio-Rad). The densities of the slots were quantified by Quantity One software (Bio-Rad).

Analysis of cell surface expressions of TF and sialic acid by flow cytometry

Subconfluent HT29-MTX cells grown in 6-well plates were treated with or without the *O*-glycosylation inhibitors for 48 hr at 37 °C. The cells were washed 3 times with PBS before being released by addition

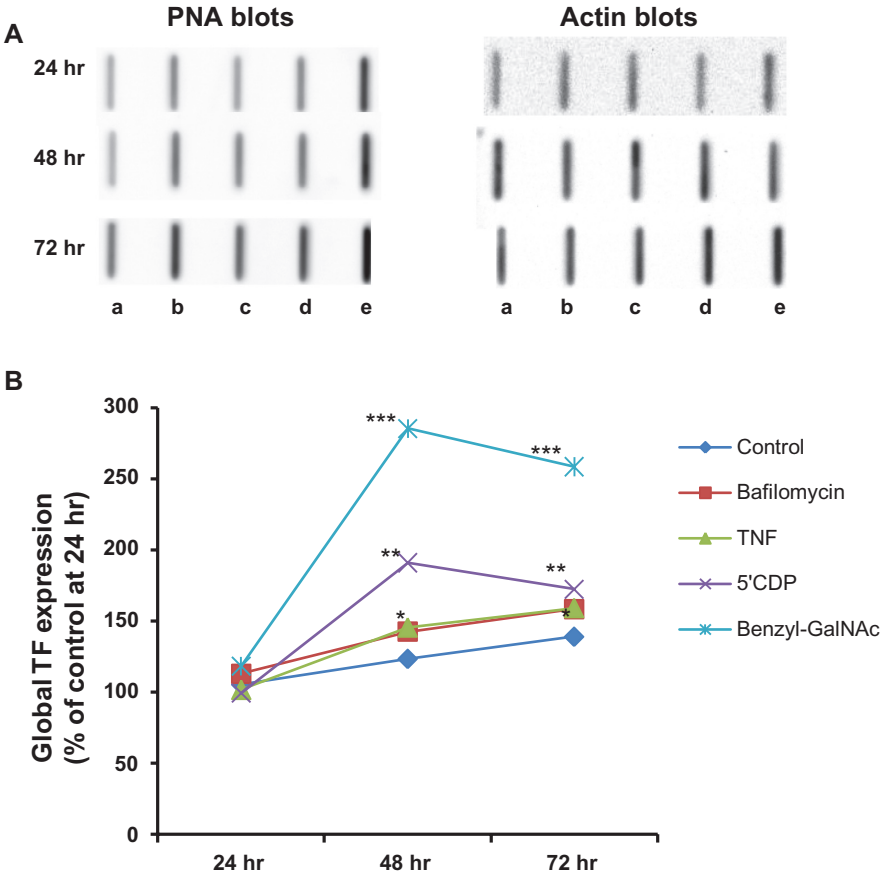

Figure 1. Effects of the *O*-glycosylation and sialylation inhibitors on total cellular expression of TF. **A**) Assessment by slot blots. HT29-MTX cells were cultured in the presence or absence of each inhibitor (a, control; b, bafilomycin; c, TNFα; d, 5'CDP; e, Benzyl-GalNAc) for 24–72 hrs before the cells were lysed and analysed by slot blots with TF-binding PNA or anti-actin antibody. **B**) Quantification of TF-expression. The densities of the slot blots from two independent experiments, each in duplicates, were analysed and the data are presented as percentage compared with the non-treated controls after normalisation. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P<0.05$.

of the Non-enzymatic Cell Dissociation Solution. The cells were washed twice with PBS, fixed in 1% paraformaldehyde for 10 min, washed 3 times with PBS and incubated with 1% BSA/PBS for 30 min before application of biotinylated- PNA or -MAL-II (1 μg/ml) in 1% BSA/PBS for 1 hr at room temperature. After washing with PBS, the cells were incubated with Avidin-FITC (1:200 dilution in 1% BSA/PBS) in dark for 1 hr. The cells were washed and resuspended in PBS and the lectin cell surface binding was analysed by flow cytometry (Becton-Dickinson, FACSVantage SE). The experiment was repeated 3 times with 10,000 gated events were recorded each time.

Hsp70 nuclear localization in response to heat stress

Subconfluent HT29-MTX cells cultured on glass coverlipes inserted in 24-well plates were incubated with or without the *O*-glycosylation inhibitors for 48 hr. The cells were then incubated at either 37 °C or 42 °C for 1 hr before fixed in 2% paraformaldehyde. The fixed cells were blocked with 5% goat serum for 1 hr at room temperature before application of the anti-Hsp70 antibody (1:200 dilution in 1% goat serum/PBS) for 2 hr. The cells were washed with PBS and applied with FITC-conjugated secondary antibody (1:500) for 1 hr in the dark. The cells were washed with PBS and mounted with a Propidium iodide (PI)-containing mounting medium (Vector). The slides were blindly labeled and the nuclear localisation of Hsp70 was visualised using an Olympus B51 fluorescent microscope. For quantification of Hsp70 nuclear localization, at least 10 individual cells for each category were randomly selected and analyzed by AQM software (Andor Technology, Nottingham, UK). Inter assay coefficient of variance was 8.3% and intra assay coefficient of variance was 8.0% for all experiments.

Statistical analysis

Unpaired 2-tailed *t* tests (StatsDirect for Windows, StatsDirect) for single comparison were used and differences were considered significant when $P < 0.05$.

Results

Effects of the agents on total cellular expressions of TF and sialic acid

The mucus-secreting human colon cancer HT29-MTX cells were incubated separately with Benzyl GalNAc, 5'CDP, TNFα or bafilomycin for various times and the total cellular expression of TF and sialic acids was assessed by slot blotting with the TF-binding peanut lectin (PNA) and the sialic acid-binding lectin, MAL-II. Bafilomycin was tested at 100 nM,[18] TNFα 2 ng/ml,[22] 5'CDP 1 mM[23] and Benzyl-GalNAc 2 mM,[23] the optimal concentration of each agent that has been shown previously to effectively inhibit cellular *O*-glycosylation. Treatment of the cells with each of the agents resulted in significant increase of global expression of TF at 48 and 72 hr, with small increases induced by bafilomycin and TNFα and large increases by 5'CDP and Benzyl-GalNAc (Fig. 1A and B). At 48 hr, 14% ($P < 0.05$), 15% ($P < 0.05$), 54% ($P < 0.01$) and 132% ($P < 0.001$) increased TF expressions, an indication of impairment of more complex *O*-glycosylation

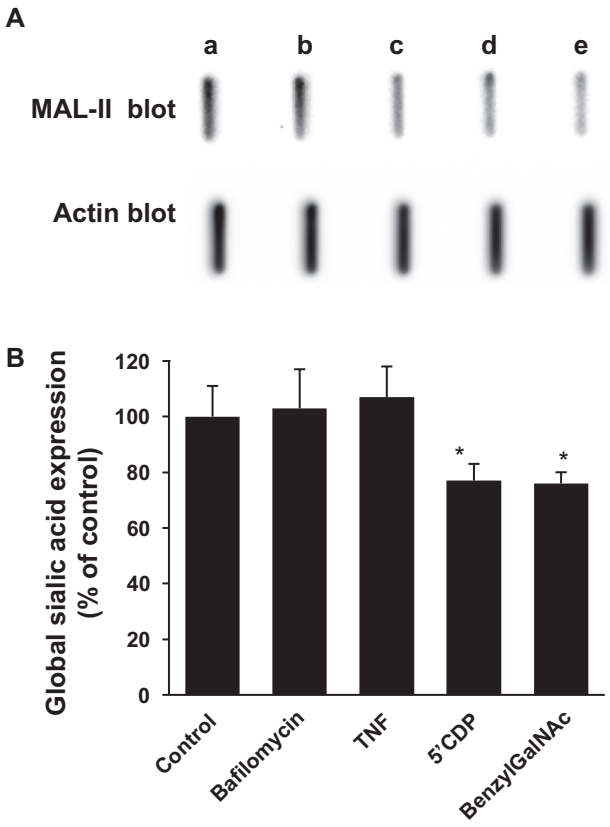

Figure 2. Effects of the *O*-glycosylation and sialylation inhibitors on total cellular sialylation. **A**) Assessment by slot blots. HT29-MTX cells were cultured in the presence or absence of each inhibitor (a, control; b, bafilomycin; c, TNFα; d, 5'CDP; e, Benzyl-GalNAc) for 48 hr before the cells were lysed and analysed by slot blots with the sialic acid-binding MAL-II or anti-actin antibody. **B**) Quantification of sialic acid expression. The densities of the slot blots from two independent experiments, each in duplicates, were analysed and the data are presented as percentage compared with the non-treated control after normalisation. *$P < 0.05$.

biosynthesis, were observed after treatment of the cells with bafilomycin, TNFα, 5'CDP and Benzyl-GalNAc, respectively. 5'CDP and Benzyl-GalNAc, but not bafilomycin nor TNFα, also caused significant reduction (23% and 24%, respectively) of global sialic acid expression after 48 hr (Fig. 2A and B). The decreased effect of Benzyl-GalNAc and 5'CDP on cellular glycosylation after 48 hr is likely due to exhaustion of these inhibitors by the growing cells.

Effects of the agents on cell surface expression of TF and sialic acid

Although the presence of each of the agents caused global increase of the expression of cellular TF, only treatment of the cells with Benzyl-GalNAc was found to induce a significant increase (322%) of the expression of TF on the cell surface (Fig. 3A and B). Benzyl-GalNAc was also the only agent that caused a significant reduction (34%) of the cell surface expression of sialic acids (Fig. 4A and B).

Effect of altered glycosylation on Hsp70 nuclear translocation upon heat stress

During normal cell culture conditions at 37 °C, Hsp70 was seen to be predominately localized in the cell cytoplasm (Fig. 5A). Upon heat stress at 42 °C for 1 hr, Hsp70 quickly moved into the

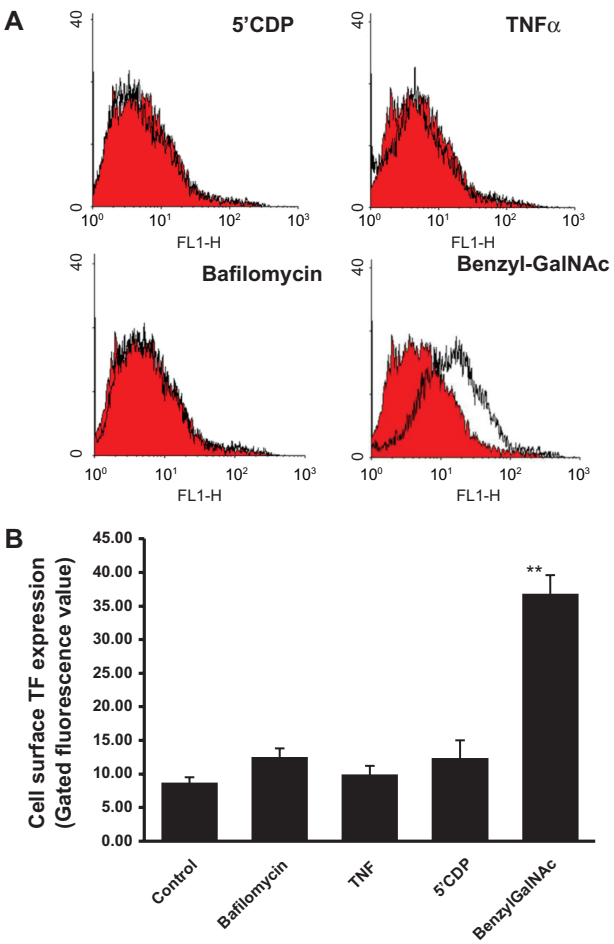

Figure 3. Effects of the *O*-glycosylation and sialylation inhibitors on cell surface expression of TF. **A**) Assessment by flow cytometry. HT29-MTX cells incubated in the presence or absence of each *O*-glycosylation inhibitor for 48 hr were analysed for cell surface TF expression with PNA-FITC followed by flow cytometry. Filled diagram, un-treated cells; open diagram, inhibitor-treated cells. **B**) Quantification of the cell surface expression of TF. The data from three independent experiments are presented as gated fluorescence values. $^{**}P < 0.01$.

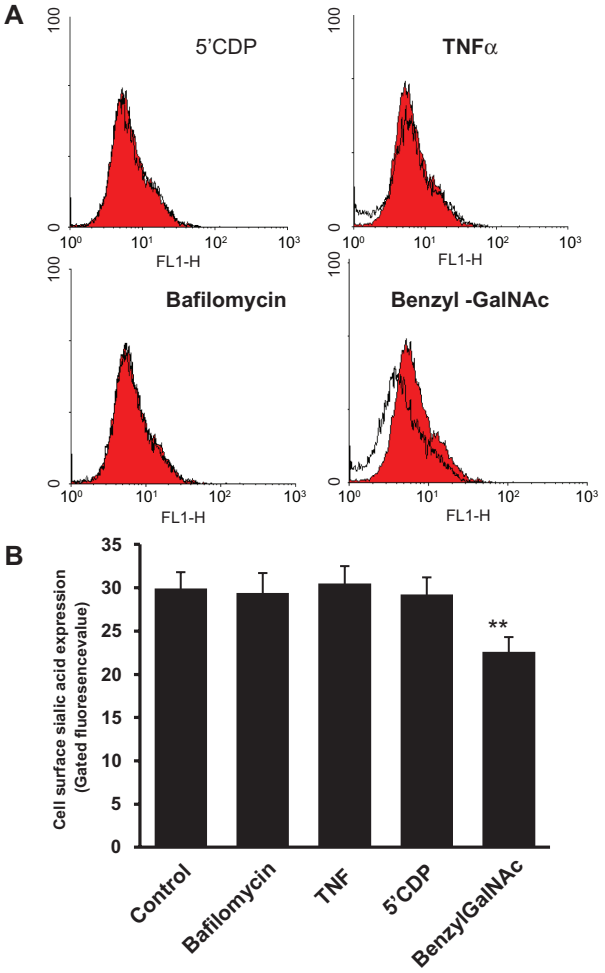

Figure 4. Effects of the *O*-glycosylation and sialylation inhibitors on sialylation of cell surface glycoproteins. **A**) Assessment by flow cytometry. HT29-MTX cells incubated in the presence or absence of each inhibitor for 48 hr were analysed for cell surface sialic acid expression with sialic acid-binding MAL-II and followed by flow cytometry. Filled diagram, un-treated cells; open diagram, inhibitor-treated cells. **B**) Quantification of the cell surface sialic acid expression. The data from three independent experiments are presented as gated fluorescence values. $^{**}P < 0.01$.

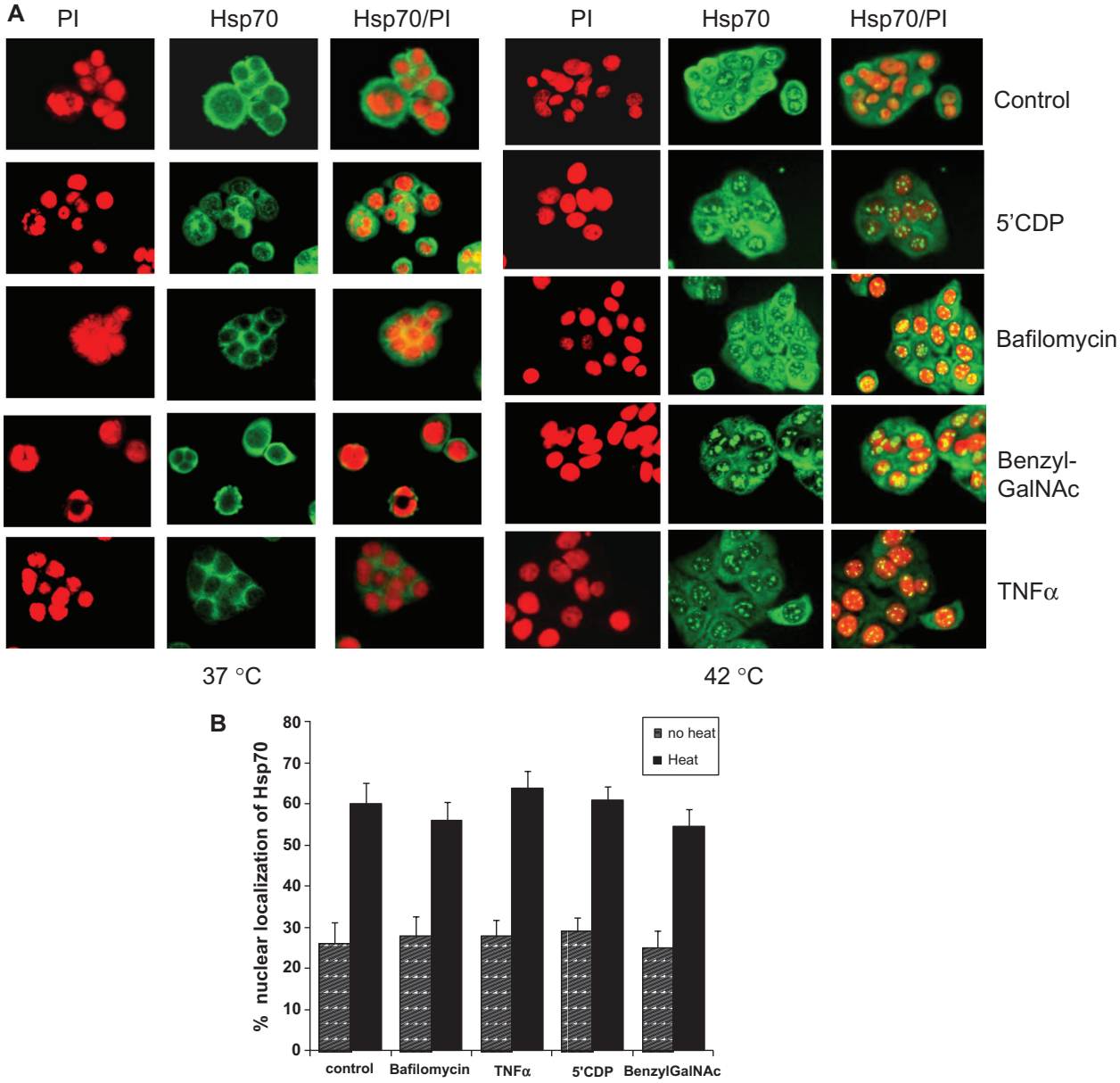

Figure 5. Effect of the *O*-glycosylation and sialylation inhibitors on nuclear import of Hsp70 upon heat stress. **A**) Hsp70 nuclear localization in response to inhibitors without and with heat stress. HT29-MTX cells cultured in the presence or absence of each of the glycosylation inhibitors for 48 hr were incubated at 37 °C or 42 °C for 1 hr before Hsp70 nuclear localization was assessed by Hsp70 immunohistochemistry. Representative images from three independent experiments are shown for each group. **B**) Quantification of Hsp70 nuclear localization. Fluorescence densities of 10 randomly selected cells in each group were quantified using AQM software. The data from three independent experiments are presented as percent nuclear fluorescence of the whole cells.

nucleus, represented by a 40% increase of nuclear fluorescence. Quantification of Hsp70 nuclear localization showed no statistical difference between the control cells and the cells pre-treated with any of the agents under test (Fig. 5B). This suggests that modification of *O*-glycosylation and/or sialylation has no effect on the NLS-dependent nuclear translocation process.

Discussion

This study showed that treatments of the mucus-secreting HT29-MTX cells with four agents that are known to affect *O*-glycosylation and/or sialylation, all caused increased expression of the core 1 precursor structure of the extended complex *O*-glycans. Treatment of the cells with Benzyl-GalNAc also induced a significant increase of the cell surface expression of

TF and a reduction of global as well as cell surface expression of sialic acids. None of these treatments however had any significant effect on Hsp70 nuclear accumulation upon heat stress. These results suggest that neither *O*-glycosylation nor sialylation are likely to be involved in NLS-dependent nuclear translocation.

It is interesting to note that although the four *O*-glycosylation inhibitors all induced an increase of global expression of cellular TF, only Benzyl-GalNAc had a demonstrable effect on the *O*-glycosylation of cell surface glycoconjugates. Earlier studies have shown that truncation of *O*-glycans of the transmembrane mucin protein MUC1, one of the few proteins known to carry unsubstituted TF structure in epithelial cancer cells,[6] reduces trafficking of intracellular MUC1 to the cell surface.[25] The secretion of MUC1 has also shown to be influenced by the *O*-glycosylation status of MUC1. The MUC1 molecules secreted by epithelial breast cancer cells were seen to carry predominately the core 2-based *O*-glycans while the membrane-bound MUC1 in the same cells were dominated by sialylated core 1 structure.[26] It is therefore possible that the reduction of cellular *O*-glycosylation induced by the inhibitors in HT29-MTX cells may have caused variably defective trafficking of glycoconjugates from the Golgi to the cell surface.

Benzyl-GalNAc is the most commonly used *O*-glycosylation inhibitor. Its inhibitory effect comes from its action as a competitive acceptor for cellular Gal-transferases and sialyl-transferases[27,28] to yield free Galβ1,3GalNAc-α-O-benzyl and sialyl-Galβ1,3GalNAc-α-O-benzyl oligosaccharides, and increase the expression of shorter *O*-glycans (e.g. GalNAcα-) on cellular and secreted proteins. The fact that Benzyl-GalNAc treatment of the cells results in an increased expression of TF and decreased expression of sialic acids on the cell surface shown in this as well as a few earlier studies[24,29] indicates that the impact of Benzyl-GalNAc on protein glycosylation may derive more from its competition for the cellular sialyl-transferase than for the Gal-transferase.

The attempts to isolate Orp150 by antibody precipitation/extraction for the assessment of Orp150 glycosylation in response to the glycosylation inhibitors were not successful due to ineffectiveness of Orp150 extraction by the current available anti-Orp150 antibodies. Sugar residues, such as glucose, fucose, mannose or N-acetyl-chitobiosides, have previously been shown able to serve as NLS-independent, nuclear translocation signals to allow transfer of BSA to the nucleus in a semi-permeabilized nuclear transport system.[30,31] The results in this study suggest that the mucin-type *O*-glycosylation and/or sialylation are unlikely to be involved in the classical NLS-dependent nuclear protein import process.

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Abbreviations

ABL, mushroom *Agaricus bisporus* lectin; Benzyl-GalNAc, GalNAc-α-O-benzyl; 5'CDP, 5'cytosine diphosphate; Hsp70, heat protein 70; MAL-II, *Maackia amurensis* lectin-II; NLS, nuclear localization sequence; Orp150, oxygen-regulated protein 150; PNA, peanut agglutinin; TF antigen, Galβ1, 3GalNAcα-Ser/Thr.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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