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# INHIBITION OF THE GLYCOSYLATION AND ALTERATION IN THE INTRACELLULAR TRAFFICKING OF MUCINS AND OTHER GLYCOPROTEINS BY GALNACa-*O*-BN IN MUCOSAL CELL LINES: AN EFFECT MEDIATED THROUGH THE INTRACELLULAR SYNTHESIS OF COMPLEX GALNACa-*O*-BN OLIGOSACCHARIDES

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## 1. ABSTRACT

To address the function of carbohydrates in mucins, GalNAcα-*O*-bn has been used in *in vivo* experiments on several human mucosal cultured cells as a potential competitor of the glycosylation of *N*-acetylgalactosamine residues. GalNAcα-*O*-bn is metabolized by glycosyltransferases expressed in the cell, and give rise to different internal derivatives starting in particular from the formation of the disaccharide Galβ1-3GalNAcα-*O*-bn. In this line, GalNAcα-*O*-bn exposure inhibits peripheral glycosylation according a cell-type specific manner. The metabolic alterations are very important in HT-29 cell line, leading to a massive accumulation of GalNAcα-*O*-bn oligosaccharide derivatives and to a strong inhibition of the terminal elongation of *O*-glycans by α2,3 sialyltransferase ST3Gal I. GalNAcα-*O*-bn treatment also induced alterations at the cellular level, exhibiting a large scale in HT-29 cells, i.e. 1) an inhibition of mucin secretion, 2) a blockade in the targeting of some membrane glycoproteins (brush border glycoproteins such as dipeptidylpeptidase IV, carcinoembryonic antigen and the mucin-like glycoprotein MUC1, and the basolateral cell adhesion molecule CD44), 3) an inhibition in the processing of lysosomal enzymes. Morphological abnormalities have been evidenced in GalNAcα-*O*-bn treated cells, in particular the accumulation of numerous intracellular vesicles in HT-29 cells. Taken together, these data suggest that *O*-glycosylation might be involved in the regulation of the targeting of *O*-glycosylproteins through carrier vesicles.

## 2. INTRODUCTION

To address the function of carbohydrates, inhibitors of glycosylation are used in *in vivo* experiments on cultured cells. Different substances are available for inhibiting the processing of *N*-glycans, such as tunicamycin, deoxynojirimycin, castanospermine, desoxymannojirimycin, swaisonine, etc (for review, 1-5). Similar tools do not exist for the biosynthesis of *O*-glycans. However, the inhibition of *O*-glycosylation processes has been obtained through the use of chemically synthesized sugar analogues. Aryl and alkyl *O*-β-D-xylosides have been developed as competitive substrates for inhibiting the elongation of *O*-linked xylose on proteoglycans (6). Similarly, aryl-*N*-acetyl-α-galactosaminides have been synthesized as potential competitors of the elongation of *O*-linked GalNAc residues on mucins (7, 8). Recently, the synthesis of *C*-glycoside analogue of benzyl-*N*-acetyl-α-galactosaminide has been reported (9).

## 3. INTRACELLULAR METABOLISATION OF GalNAcα-*O*-bn

Benzyl-*N*-acetyl-α-galactosaminide (GalNAcα-*O*-bn) was particularly used on human mucosal cells which can be grown in long-term culture (10), in the aim of studying the function of the *O*-glycosylation of mucins. Investigations in different cell-types (LS174T, Kato III, Caco-2 and HT-29 cell lines) showed that this derivative comes through the membrane and undergoes intracellular metabolisation (7, 8, 11, 12). The disaccharide Galβ1-3GalNAcα-*O*-bn accounts for a main GalNAcα-*O*-bn derivatives synthesized intracellularly, but many other different derivatives were also identified in the different cell types by metabolic labeling. Complex benzyl-oligosaccharides are synthesized inside the cells (up to 6-7 sugars in Caco-2 cells, 8-9 sugars in Kato III cells (11),

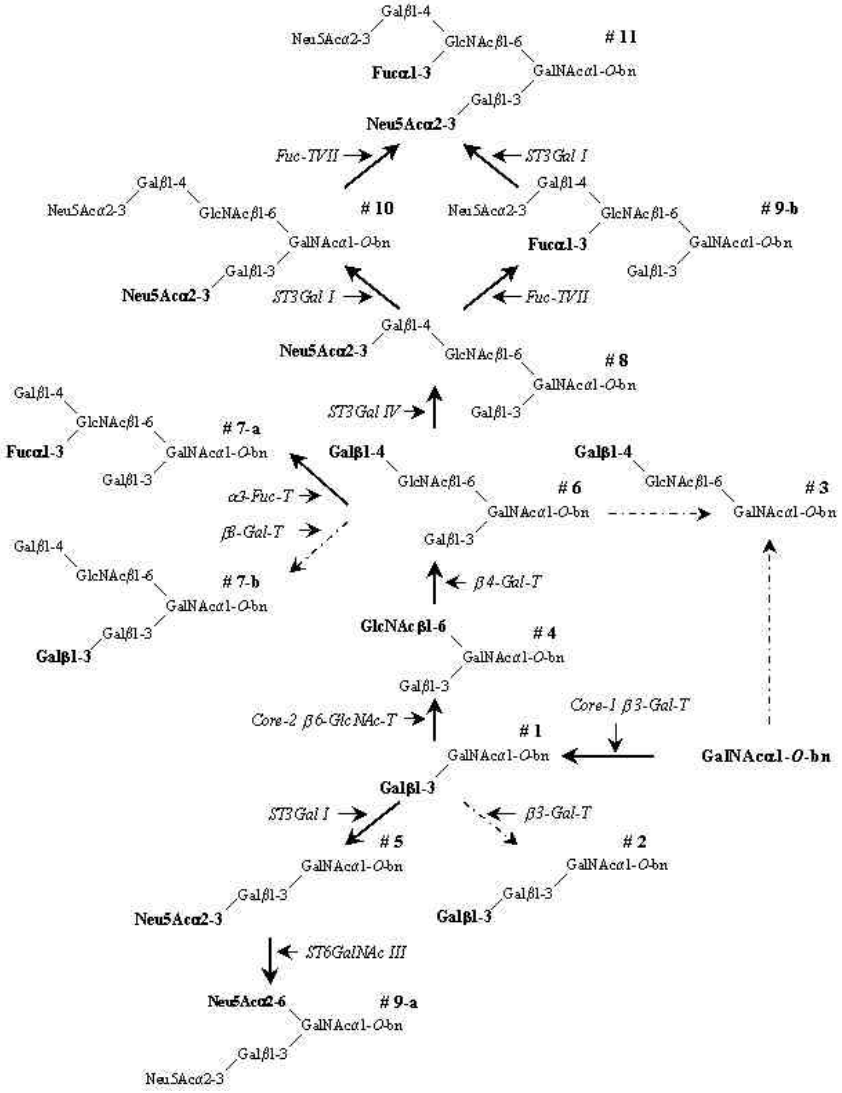


**Figure 1**: Structure and hypothetical biosynthesis scheme of the main GalNAcα-*O*-bn derivatives accumulated in HT-29 $G^-$ cells. The indicated glycosyltransferases have been proposed according to their known specificity in the biosynthesis of human oligosaccharides. The sugar residues transferred are indicated in bold letters. Arrows leading to compound # 2, 3 and 7b are dotted to indicate that the pathway is not clearly defined.

and 6 sugars in HT-29 cells (13)).

The identification of synthesized GalNAcα-*O*-bn derivatives was investigated in HT-29 cells. In the first experiments by reverse phase HPLC, two GalNAcα-*O*-bn derived oligosaccharides were identified after a time exposure of 24 hours: the disaccharide Galβ1-3GalNAcα-*O*-bn and the trisaccharide Neu5Acα2-3Galβ1-3GalNAcα-*O*-bn (12). Then, after a permanent exposure, using MALDI-TOF, GC/MS and $^1$H-NMR analyses, eleven different GalNAcα-*O*-bn derivatives were identified and are shown under a synthesis pattern in Figure 1 (13). They could be divided into three families of compounds, according to the addition of further residues on GalNAc: Galβ1-3 and / or GlcNAcβ1-6 or Neu5Acα2-6. The simplest benzyl-oligosaccharide was Galβ1-3GalNAcα-*O*-bn (compound # 1). As shown in Figure 1, the Galβ1-3 branch could be substituted either by another Galβ1-3 residue (compound # 2) or by a Neu5Acα2-3 residue (compounds # 9-a, 10, 11). In addition, GalNAc residue of Galβ1-3GalNAcα-*O*-bn motif could also be substituted by a GlcNAcβ1-6 (compound # 4). This GlcNAc residue constituted the starting point of a series of compounds, firstly always comprising an additional Galβ1-4 (compound # 6) that could be sialylated by Neu5Acα2-3 (compounds # 9-b, 10 and 11). GlcNAc residue could be itself substituted by Fucα1-3 (compounds # 9-b and 11), forming sialyl-$Le^x$ motif. Compound # 3 was unambiguously identified as Galβ1-4GlcNAcβ1-6GalNAcα-*O*-benzyl, a member of the core-6 series of *O*-glycans.

The structure of the oligosaccharide side chains of the mucins synthesized by mucin-secreting HT-29 cells (HT-29 MTX subpopulation, 14) have been determined and the major structure found was the trisaccharide, sialyl T, i.e. Neu5Acα2-3Galβ1-3GalNAc. It may be stressed that this structure was also found on GalNAcα-*O*-bn, i.e. compound # 5, and furthermore, this compound accounted for a derivative synthesized in a

relatively high amount. Consequently, GalNAcα-*O*-bn carries oligosaccharide structures similar as in mucins. However, complex fucosylated GalNAcα-*O*-bn derivatives are also synthesized in differentiated HT-29 cells (of enterocytic phenotype, HT-29 G- cells, (13) and also of mucin-secreting phenotype, HT-29 MTX cells, unpublished data) despite the fact that no fucosylated oligosaccharide structure was detected in the mucins of HT-29 MTX cells (15). Furthermore, among all the GalNAcα-*O*-bn derivatives, only the oligosaccharide structures of the compounds # 5, 8, 9a and 10 were previously found in HT-29 MTX mucins. This observation finally shows that the elongation of GalNAcα-*O*-bn does not strictly reflects the elongation of GalNAc residues linked to apomucins, and suggests that the specificity of glycosylation of mucins is also relevant to the peptide backbone of mucins.

The amount of GalNAcα-*O*-bn derivatives accumulated in HT-29 cells is very high: 1 mg hexose / mg protein. In contrast, no accumulation of either GalNAcα-*O*-bn derivative occurred in Caco-2 cells (13). This observation indicated a lower rate of biosynthesis and/or a higher rate of degradation of GalNAcα-*O*-bn derivatives in Caco-2 cells than in HT-29 cells. Obviously, the fate of GalNAcα-*O*-bn can be very different according to the cell type.

## 4. INHIBITION OF SEVERAL GLYCOSYLTRANSFERASES IN HT-29 CELLS

Figure 1 shows the possible pathway for the biosynthesis of the different GalNAcα-*O*-bn oligosaccharide derivatives by the known glycosyltransferases. It was interesting to note that the biosynthesis of GalNAcα-*O*-bn oligosaccharides was perfectly in agreement with the commonly described *O*-glycan biosynthesis pathways and with the substrate specificity determined for the different enzymes in *in vitro* assays (16). GalNAcα-*O*-bn treatment could be therefore a convenient tool to determine the *O*-glycosylation pattern of cultured cells. All the glycosyltransferases involved in the synthesis of these derivatives (Table 1) might thus be potentially inhibited for the substitution of endogenous substrates.

As previously mentioned, GalNAcα-*O*-bn constitutes a very good substrate for UDP-Gal : GalNAc β1,3 galactosyltransferase (core-1 β3-Gal-T) in different types of cultured cells, including HT-29 cells (7, 8, 12). A cDNA sequence encoding a human core-1 β3-Gal-T recently appeared in the databases (Ju, unpublished data) but the enzyme has not been precisely characterized to date. The formed Galβ1-3GalNAcα-*O*-bn also behaves as an acceptor substrate for several other glycosyltransferases: Galβ1-3GalNAc α2,3-sialyltransferases : ST3Gal I (17) and ST3Gal II (18, 19) and core-2 β1,6-*N*-acetyl-glucosaminyltransferase I (20) and II (21). The oligosaccharide structure Neu5Acα2-3Galβ1-3-GalNAc (compound # 5), found in a high amount, is synthesized through the catalytic action of ST3Gal I which is highly expressed in HT-29 cells (15). Furthermore, some of these firstly processed compounds are further elongated. For instance, Neu5Acα2-3Galβ1-3GalNAcα-*O*-bn can be substituted by Neu5Acα2-3Galβ1-3GalNAc (sialyl to GalNAc) α2,6-sialyltransferases ST6GalNAc III (22) and IV (23), leading to the synthesis of the disialylated form of T-antigen (compound # 9-a). In this connection, neither Neu5Acα2-6GalNAcα-*O*-bn (sialyl-Tn) nor Galβ1-3[Neu5Acα2-6]GalNAcα-*O*-bn were observed. This observation fits well with our knowledge of the substrate specificity of ST6GalNAc I (24) and ST6GalNAc II (25), the sialyltransferases involved in the biosynthesis of these structures, since both require a peptide aglycon to be active. GlcNAcβ1-6[Galβ1-3]GalNAcα-*O*-bn (compound # 4) can be further elongated onto GlcNAcβ1,6-branch by the sequential addition of a β1,4-linked Gal residue (compound # 6) and of a α2,3-linked Neu5Ac residue (compound # 8) transferred by one of the human β1,4-galactosyltransferases (26) and a Galβ1-4GlcNAc α2,3-sialyltransferase, respectively. Two different Galβ1-4GlcNAc α2,3-sialyltransferases: ST3Gal IV (27, 28) and VI (29) have been characterized and we previously showed that ST3Gal IV is highly expressed in HT-29 cells (15). Compound # 8 is further the precursor of compounds # 9-b (the sialyl-Lewis$^X$ epitope) and # 10. These two compounds reflect the catalytic action of either a Neu5Acα2-3Galβ1-4GlcNAc α1,3-fucosyltransferase, probably Fuc-TVII which specifically synthesizes the sialyl-Lewis$^x$ epitope (30, 31), or ST3Gal I, respectively. It is clear that in HT-29 cells the biosynthesis of sialyl-Lewis$^X$ determinant requires the α2,3-sialylation of Galβ1-4GlcNAc terminal sequence prior the α1,3-fucosylation (32). Finally, the more complex GalNAcα-*O*-bn derivative (compound # 11) which contains both a sialyl-Lewis$^x$ epitope onto the β1,6-branch and a α2,3-linked Neu5Ac residue to the terminal Gal of core 1, reflects the action of both ST3Gal I and Fuc-TVII. In parallel with that main biosynthetic pathway of GalNAcα-*O*-bn derivatives, several other minor compounds were observed. As an example, compound # 6 may be used as an acceptor substrate by an α3-fucosyltransferase different from Fuc-TVII leading to compound # 7-a which contains a Lewis$^x$ epitope onto the β1,6-branch. Several α3-fucosyltransferases such as Fuc-TIV, Fuc-TV, Fuc-TVI (33), or the newly cloned Fuc-TIX (34) could be responsible for the biosynthesis of this compound.

For some compounds (# 2, 3, and 7b), the biosynthesis pattern could not be clearly defined with the known human glycosyltransferases. Compounds # 1 and 6 apparently serve as acceptor substrates for a putative β1,3-galactosyltransferase to generate the compounds # 2 and 7-b, respectively. However the Galβ1-3Galβ1- terminal sequence has never been observed in oligosaccharides from mammalian mucins and no UDP-Gal : Galβ1-3GalNAc β1,3galactosyltransferase has been detected in mammalian tissues or cells till now (16). But it can be hypothesized that compound #2 is formed by the action of the β1,3-galactosyltransferase acting on glycosaminoglycans. Surprisingly, even if most compounds described here derive from Galβ1-3GalNAcα-*O*-bn, we could identify the structure of Galβ1-4GlcNAcβ1-6GalNAcα1-*O*-bn (compound # 3) at a significant level. This compound could correspond to a degradation of compound # 6 by the action of a specific galactosidase, all the more than the disaccharide GlcNAcβ1-6GalNAcα-*O*-bn could not be detected.

**Table 1.** Enzymes potentially inhibited by GalNAc-α-*O*-bn treatment in HT-29 cells

| Enzymes | | EC number | Accession # | References |
|---|---|---|---|---|
| core-1 β3-Gal-T | UDP-Gal : GalNAc-R β1,3-galactosyltransferase | EC 2.4.1.122 | AF155582 | Ju, unpublished data |
| core-2 β6-GlcNAc-T-I | UDP-GlcNAc : Galβ1-3GalNAc-R (GlcNAc to GalNAc) β1,6-N-acetylglucosaminyltransferase | EC 2.4.1.102 | M97347 | 20 |
| core-2 β6-GlcNAc-T-II | UDP-GlcNAc : Galβ1-3GalNAc-R (GlcNAc to GalNAc) β1,6-N-acetylglucosaminyltransferase | EC 2.4.1.- | AF102542 | 21 |
| β4-Gal-T | UDP-Gal : GlcNAc-R β1,4-galactosyltransferase | EC 2.4.1.38 | X55415<br>AF038660<br>AF038661<br>AF038662<br>AF038663<br>AF038664 | 26 |
| Fuc-TIV[1] | GDP-Fuc : Galβ1-4GlcNAc (Fuc to GlcNAc) α1,3-fucosyltransferase | EC 2.4.1.- ; (myeloid enzyme) | M58596 | 45 |
| Fuc-TV | GDP-Fuc : Galβ1-4GlcNAc (Fuc to GlcNAc) α1,3-fucosyltransferase | EC 2.4.1.- | M81485 | 46 |
| Fuc-TVI | GDP-Fuc : Galβ1-4GlcNAc (Fuc to GlcNAc) α1,3-fucosyltransferase | EC 2.4.1.152 ; (plasma enzyme) | L01698 | 47 |
| Fuc-TIX | GDP-Fuc : Galβ1-4GlcNAc (Fuc to GlcNAc) α1,3-fucosyltransferase | EC 2.4.1.- | AB023021 | 34 |
| Fuc-TVII | GDP-Fuc : Neu5Acα2-3Galβ1-4GlcNAc α1,3-fucosyltransferase | EC 2.4.1.- | X78031<br>U08112 | 30<br>31 |
| ST3Gal I | CMP-Neu5Ac : Galβ1-3GalNAc α2,3-sialyltransferase | EC 2.4.99.4 | L29555 | 17 |
| ST3Gal II | CMP-Neu5Ac : Galβ1-3GalNAc α2,3-sialyltransferase | EC 2.4.99.- | X96667<br>U63090 | 18<br>19 |
| ST3Gal IV | CMP-Neu5Ac : Galβ1-4GlcNAc α2,3-sialyltransferase | EC 2.4.99.- | L23767<br>NM006278 | 27<br>28 |
| ST3Gal VI | CMP-Neu5Ac : Galβ1-4GlcNAc α2,3-sialyltransferase | EC 2.4.99.- | NM006100 | 29 |
| ST6GalNAc I | CMP-Neu5Ac : R-GalNAcα1-O-Ser/Thr α2,6-sialyltransferase | EC 2.4.99.3 | Y11339<br>Y11340 | 24 |
| ST6GalNAc II | CMP-Neu5Ac : $(Neu5Ac\alpha2\text{-}3)_{0\text{-}1}Gal\beta1\text{-}3GalNAc\alpha1$-O-Ser/Thr α2,6-sialyltransferase | EC 2.4.99.- | AJ251053 | 25 |
| ST6GalNAc III | CMP-Neu5Ac : Neu5Acα2-3Galβ1-3GalNAc α2,6-sialyltransferase | EC 2.4.99.7 | | 48 |
| ST6GalNAc IV | CMP-Neu5Ac : Neu5Acα2-3Galβ1-3GalNAc α2,6-sialyltransferase | EC 2.4.99.- | AJ271734 | 22 |

1 : α3-Fuc-T: GDP-Fuc: Galβ1-4GlcNAc (Fuc to GlcNAc) α1,3-fucosyltransferase, including Fuc-TIV (myeloid enzyme) *FUT4*-encoded α1,3-fucosyltransferase, Fuc-TV. 2: *FUT5*-encoded α1,3-fucosyltransferase, Fuc-TVI (plasma enzyme, EC 2.4.1.152) *FUT6*-encoded α1,3-fucosyltransferase, and Fuc-TIX *FUT9*-encoded α1,3-fucosyltransferase.

In conclusion, GalNAcα-*O*-bn treatment of HT-29 cells can potentially inhibit many glycosyltransferases involved in the biosynthesis of *O*- and / or *N*-glycans (Table 1).

## 5. MORPHOLOGICAL CHANGES

Morphological changes were observed after a long time exposure to GalNAcα-*O*-bn. In LS174T cells, the formation of intercellular cysts was detected from day 3 of exposure, increased thereafter and reached a steady number after one week of exposure (7). The appearance of pleomorphic vacuoles and an increase in lysosomes was also observed. Strong morphological changes were found after permanent exposure of HT-29 cells to GalNAcα-*O*-bn: a dramatic swelling of the cells, and an accumulation of numerous intracytoplasmic vesicles (Figure 2 a, b) (35). In contrast, such changes were not observed for Caco-2 cells. Taken together, these observations evidenced the cell-type specificity of the cell reaction to GalNAcα-*O*-bn. Differences in the structure and / or level of accumulated GalNAcα-*O*-bn metabolites may account for the specific behavior regarding cell morphology. In HT-29 cells, several data support the idea that the GalNAcα-*O*-bn oligosaccharides accumulate inside the numerous intracytoplasmic vesicles: i) these derivatives are recovered in the glycolipid phase of Folch extraction (13), ii) the presence of some glycan epitopes of GalNAcα-*O*-bn derivatives could be shown in intracytoplamic vesicles (unpublished data).

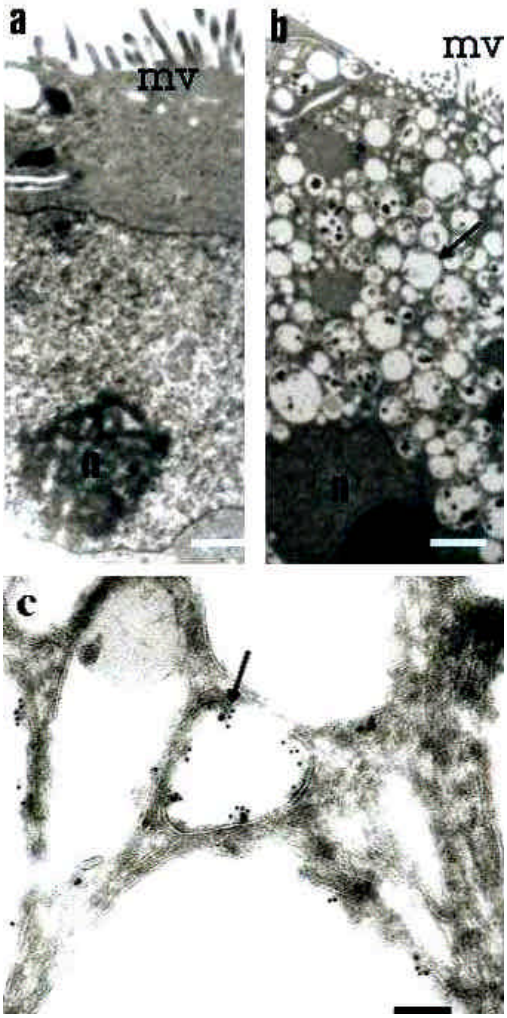


**Figure 2.** Ultrastructural morphology and localization of DPP-IV in control and GalNAcα-*O*-bn treated HT-29 $G^-$ cells. **(a, b)** Standard transmission electron microscopy of sections perpendicular to the bottom of the flask showing the apical microvilli in both control (a) and GalNAcα-*O*-bn treated cells (b), and the very numerous vesicles (arrow) in GalNAcα-*O*-bn treated cells (b). **(c)** Immunogold staining of DPP-IV in GalNAcα-*O*-bn treated HT-29 $G^-$ cells. The gold particles are associated with the cytoplasmic vesicles. Bars: (a) 0.68 µm, (b) 1.95 µm, (c) 0.13 µm. mv: apical microvilli.

## 6. EFFECT OF GalNAcα-*O*-bn ON SECRETED MUCINS

### 6.1 Glycosylation of mucins

First experiments on mucin producing cancer cell lines were carried out by Kuan *et al.* (7). Mucin labeling with [$^3$H]-glucosamine revealed that a 24 hour exposure to benzyl-, phenyl-, and p-nitrophenyl-*N*-acetyl-α-galactosaminide inhibited the glycosylation of mucins in LS174T cells (7). GalNAcα-*O*-bn was further shown to inhibit mucin glycosylation in other cell lines (Kato III, Caco-2 and HT-29) after 24 to 48 hours incubation, and studies were undertaken in order to bring information about the alterations induced in these different cell lines, using chemical analysis and / or immunoassays with lectins and glycan specific antibodies (8, 11, 36, 15). In all cell lines, GalNAcα-*O*-bn treatment induced an increase in the expression of Tn antigen (GalNAc-Thr / Ser), but also, an increase in the expression of T antigen (Galβ1-3GalNAc). The latter observation was surprising because GalNAcα-*O*-bn was initially used as a competitive inhibitor of the activity of core-1 β3-Gal-T through the formation of the disaccharide Galβ1-3GalNAcα-*O*-bn. In fact, GalNAcα-*O*-bn does not efficiently inhibited core-1 β3-Gal-T in vivo on the different cultured cells because the activity of this enzyme is very high in these cells and can transfer a Gal residue on both the endogenous (mucins) and exogenous (GalNAcα-*O*-bn) substrates. In contrast, the high amount of the formed disaccharide Galβ1-3GalNAcα-*O*-bn acts as an efficient inhibitor of the elongation of the T antigen structures of mucins (8). In parallel to the increased expression of core region carbohydrate antigens Tn and T, GalNAcα-*O*-bn was found to decrease the expression of peripheral carbohydrate antigens, this time according a cell line-specific manner: sialyl $Le^x$ and sulfomucin in LS174T cells (8), sialyl $Le^x$ in Kato III cells (11), terminal fucose (e.g. H-antigen) in Caco-2 cells (11), and sialyl T antigen in HT-29 MTX cells (36). This specific effect of GalNAcα-*O*-bn upon peripheral glycosylation is likely dependent upon the glycosyltransferase expression pattern in each cell type.

A chemical analysis of mucins synthesized in presence of GalNAcα-*O*-bn was investigated in mucin-secreting HT-29 MTX cells. This analysis was carried out after a short time exposure (24h) on differentiated cells at a late post-confluence state (day 21) (35), and also after a permanent exposure of cells from their undifferentiated state (day 2 after seeding) up to late confluence (day 21) (15). Carbohydrate compositions are shown as molar ratios to GalNAc (Table 2) in comparison to the carbohydrate composition of mucins of control cells. The short time exposure to GalNAcα-*O*-bn particularly induced a dramatic decrease (by 13-fold) in the relative amount of sialic acid. No change was found in the relative amount of Gal residues. This result showed that the sialylation of mucins was primarily affected in this condition. After the permanent exposure, the decrease in the sialic acid content was not so marked (by 2.6-fold). In addition, a decrease in the content of Gal residues became apparent. Consequently, in the permanent exposure, the inhibition of sialylation was lower, and an inhibition of galactosylation occurred.

Structural investigations have determined that oligasaccharides of HT-29 MTX mucins consist of short sialylated structures (two to seven residues), mainly of core types 1 (57.5 %) and 2 (20.5 %), and that incorporation of sialic acid occurred primarily via an α2,3-linkage to a terminal Gal residue. The major structure was the monosialylated trisaccharide of core type 1, i.e. Neu5Acα2-3Galβ1-3GalNAc (41 %) (15). Consequently, the inhibition of the incorporation of Gal and/or Neu5Ac residues in HT-29 MTX mucins by GalNAcα-*O*-bn might be related to an inhibition of core-1 β3-Gal-T and / or ST3Gal I glycosyltransferases.

### 6.2. Secretion of mucins

An inhibition of mucin secretion was found in mucin-secreting HT-29 MTX cells, but not in other cell lines (7, 8, 11, 15, 36). Metabolic labeling experiments

**Table 2**. Carbohydrate composition of mucins from control HT-29 MTX cells and GalNAcα-*O*-bn-treated HT-29 MTX cells

| Carbohydatres * | Control HT-29 MTX mucins | GalNAcα-O-bn-treated HT-29 MTX mucins | |
|---|---|---|---|
| | | short exposure (24h) | permanent exposure |
| Galactose | 1.4 | 1.4 | 0.9 |
| GalNAc | = 1 | = 1 | = 1 |
| GlcNAc | 0.6 | 0.5 | 0.4 |
| Sialic acid | 1.3 | 0.1 | 0.5 |

* expressed in molar ratio to GalNAc

with [$^3$H] threonine on differentiated HT-29 MTX cells over 24 hours in presence of GalNAcα-*O*-bn showed that the secretion of mucins was inhibited by 51 % (36). Analysis of mucin secretion after permanent exposure to GalNAcα-*O*-bn was investigated in HT-29 MTX cells by metabolic labeling from day 3 to day 21. From day 7 onwards, control HT-29 MTX cells began to secrete high amounts of mucins. Maximal secretion was observed at days 9-11 and then the level of secreted mucins decreased progressively from day 13 to day 21. In contrast, the mucin secretion of treated cells remained at a very low level at day 7 and even gradually decreased up to day 21.

Regarding the strong inhibition of the constitutive secretion of mucins in HT-29 MTX cells, the effect of GalNAcα-*O*-bn upon the secretory response was then examined using the combined action of $Ca^{2+}$ ionophore and PMA, or of $Ca^{2+}$ ionophore and forskolin. Whatever the combination of secretagogues used, the secretory response of GalNAcα-*O*-bn treated cells was dramatically reduced in comparison to control cells. In line with the knowledge that MUC5AC is the major gene expressed in HT-29 MTX cells, the inhibition of both constitutive and secretagogue-induced MUC5AC secretion was specified by Western blot and ELISA experiments using an anti-apomucin (MUC5AC) antibody (15).

These results showed that, although the glycosylation of mucins was altered in the different cell-types, their secretion appeared only altered in HT-29 cells. This observation could be relevant to the cell-type specific effect of GalNAcα-*O*-bn upon peripheral glycosylation of mucins and / or the particular accumulation of GalNAcα-*O*-bn derivatives in the HT-29 cell-type.

## 7. EFFECT OF GalNAcα-*O*-bn ON MEMBRANE GLYCOPROTEINS

### 7.1. Glycosylation of membrane glycoproteins

In addition to secreted mucins, the effect of GalNAcα-*O*-bn exposure upon the glycosylation of other glycoproteins was also analyzed particularly for brush-border associated glycoproteins and cell adhesion molecules in HT-29 cells. Different brush-border associated glycoproteins, i.e. dipeptidylpeptidase-IV (DPP-IV), the mucin-like glycoprotein MUC1, and carcinoembryonic antigen (CEA), and basolateral glycoproteins i.e. integrins, gp525, and CD44 were examined for their glycosylation (35, 37, 38). All these glycoproteins were found contain a terminal sialylation by α2,3-linked sialic acid. After permanent GalNAcα-*O*-bn treatment, this terminal α2,3-sialylation was found to be inhibited for some glycoproteins (DPP-IV, MUC1, CEA, CD44) but not for others (integrin β4 and α6, gp525). Among the glycoproteins with inhibited sialylation, the appearance of T antigen expression could be shown for three glycoproteins (DPP-IV, MUC1, and CD44). This antigen is a structure specifically *O*-linked to threonine or serine residues, and its substitution by α2,3-linked sialic acid is only obtained through the enzymatic activity of ST3Gal I. These observations evidenced that GalNAcα-*O*-bn treatment primarily inhibited the elongation of *O*-glycans by ST3Gal I in HT-29 cells. This conclusion is further supported by the observation that ST3Gal I is also highly expressed in enterocytic HT-29 cells as in mucus-secreting HT-29 cells. In contrast, *N*-glycosylated glycoproteins appeared to show variable sensitivity to GalNAcα-*O*-bn treatment. Indeed, we previously mentioned that sialytransferases involved in the biosynthesis of *N*-glycans could be also competitively inhibited by metabolites of GalNAcα-*O*-bn, and ST3Gal IV is also a sialyltransferase highly expressed in HT-29 cells.

The effect of GalNAcα-*O*-bn was also studied in other cell types using short-time exposure. Alfalah *et al.* (39) reported an inhibition of *O*-glycosylation of the brush border glycoprotein sucrase-isomaltase in Caco-2 cells, Nakano et al (40) an inhibition of the sialylation of CD44 in B16BL6 melanoma cells, and Ait Slimane *et al.* (41) an inhibition of the sialylation of a soluble mouse DPP-IV form in Caco-2 and MDCK cells. However as for secreted mucins, the changes in the glycosylation of membrane glycoproteins are also dependant upon the glycosyltransferase expression pattern of the cell (38).

### 7.2. Intracellular trafficking of membrane glycoproteins

In addition to the inhibition of mucin secretion in HT-29 MTX cells, we showed that GalNAcα-*O*-bn also blocked the apical targeting of several membrane brush border glycoproteins: a transmembrane glycoprotein (DPP-IV) (Figure 2 c), a glycoprotein anchored through glycosylphosphatidylinositol moiety (CEA), and a mucin-like membrane glycoprotein (MUC1) in differentiated HT-29 cells of mucin-secreting or enterocytic phenotype (35, 38). These glycoproteins no more reached their membrane apical localization, but remained stored in the cytoplasm. This finding raised the hypothesis of a role of Neu5Acα2,3-glycosylation in the apical targeting in differentiated HT-29 cells.

In the line of such hypothesis, no change was found for the basolateral distribution of gp120 (35) and gp525 in GalNAcα-*O*-bn treated HT-29 cells (38). In contrast, we recently reported an alteration of the

basolateral distribution of CD44. This different behavior for CD44 might be related to the fact that CD44 is both *N*- and *O*- glycosylated, whereas gp120 and gp525 are only *N*-glycosylated. Indeed, as mentioned in the previous subsection, GalNAcα-*O*-bn was found to inhibit the sialylation of CD44 but not that of gp525 and of integrins (37). These data might suggest a role of *O*-glycans in intracellular trafficking of *O*-glycosylproteins. In this line, O-glycosylated stalk region of sucrase-isomaltase was recently reported to be involved in prerequisite determinants for apical targeting via sphingolipid-cholesterol microdomains (42).

In Caco-2 cells, GalNAcα-*O*-bn was also reported to induce a selective alteration in the apical targeting of the brush border glycoproteins sucrase-isomaltase (39) and DPP-IV (41). However, we recently showed that long-term GalNAcα-*O*-bn treatment of Caco-2 cells did not induce such a massive alteration in the intracellular trafficking as in HT-29 cells. In particular, no intracellular accumulation of glycoproteins occurred, but only a reduced expression at the apical cell surface (38). This observation can be related to the differences induced by GalNAcα-*O*-bn treatment in the glycosylation of membrane glycoproteins between the two cell-types and / or the specific accumulation of GalNAcα-*O*-bn derivatives in HT-29 cells.

## 8. EFFECT OF GalNAcα-*O*-bn ON LYSOSOMAL EMZYME PROCESSING

Recent work showed that the processing of lysosomal enzymes was altered in GalNAcα-*O*-bn treated HT-29 MTX cells (37). In particular, α-glucosidase was shown to accumulate as an immature molecular species in a post-trans-Golgi network (TGN) compartment. Incomplete maturation of the enzyme procathepsin D was also found. All these data suggested an alteration in the intracellular trafficking via endosomal/lysosomal compartments. Such hypothesis agrees with the inhibition of the degradation of GalNAcα-*O*-bn derivatives in these cells (13).

## 9. PERSPECTIVES

Altogether, these data show that GalNAcα-*O*-bn treatment differently affects the glycosylation of secreted mucins and membrane glycoproteins according to the cell type. Finally, this derivative does not behave primarily as an inhibitor of the elongation of *O*-linked GalNAc residues as previously expected, but as an inhibitor of peripheral glycosylation, particularly concerning the elongation of T antigen Galβ1-3GalNAc. The changes in peripheral glycosylation are related to the cell expression pattern of glycosyltransferases and are mediated through the intracellular synthesis of GalNAcα-*O*-bn oligosaccharides by glycosyltransferases. The HT-29 cell-type is peculiar in this latter field, being able to synthesize and accumulate a series of complex GalNAcα-*O*-bn oligosaccharide derivatives.

At the cellular level, the changes induced by GalNAcα-*O*-bn also deeply differ according to the cell type. Generally, alterations in the intracellular trafficking occurred, particularly concerning the apical targeting, suggesting that some steps could be regulated by *O*-glycosylation. But it must be stressed that the HT-29 cell reaction is spectacular. The strong alterations in the intracellular trafficking are very likely in close relation to the presence of numerous vesicles in the cytoplasm. A first possible mechanism to explain the specific phenotype acquired by HT-29 cells would be a blockade in the advance of carrier vesicles coming from the trans-Golgi network. This would lead to an intracellular accumulation of mistargeted glycoproteins in cellular compartments probably in the route of late endosomes/lysosomes. As another possible mechanism, a blockade in the recycling of endocytosed glycoproteins was suggested (37). Finally, the characterization of accumulated vesicles will help to elucidate the mechanisms by which GalNAcα-*O*-bn induces the expression of such phenotype in HT-29 cells.

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**Footnotes:** The nomenclature of sialyltransferases is based on Tsuji *et al.* (43), the nomenclature of fucosyltransferases is based on Oriol *et al.* (44) and the nomenclature of the other enzymes involved in O-glycan biosynthesis is based on Brockhausen (16)

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