O-Glycan inhibitors generate aryl-glycans, induce apoptosis and lead to growth inhibition in colorectal cancer cell lines

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Introduction

The surface membranes of mammalian cells are rich in glycoconjugates which mediate many cellular events and are intimately involved in cell–cell and cell–matrix interactions (Van den Steen et al. 1998). Glycosylation of cellular and membrane proteins includes N- and O-linked glycoproteins, proteoglycans, and glycosylphosphatidylinositol (GPI) anchors (Schachter and Brockhausen 1992; Fuster and Esko 2005). The mucin-type α-glycan chains are linked to serine and threonine through an α-O-glycosyl linkage to N-acetylgalactosamine in well-defined core structures (Corfield et al. 2001; Linden et al. 2008). The mucin-type O-glycans are abundant as components of the mucosal protective barrier and are also found in a variety of other proteins (Corfield et al. 2001; Thornton et al. 2008). Mucin O-glycans play a role in membrane trafficking (Gouyer et al. 2001; Huet et al. 2003; Prescher et al. 2004; Dube and Bertozzi 2005) and cell signalling through multivalent protein–glycan networks mediated by galectins (Brewer et al. 2002; Gabius 2006), implicating roles in cell and tissue development and viability.

The glycobiology of immune function has been demonstrated through controlled glycosylation of endogenous lectin ligands. Glycosylation of CD45 on immature thymocytes and activated T cells is regulated through the action of the glycosyltransferases α-2,6-sialyltransferase 1 (ST6GAL1) and core 2 β-1,6-N-acetylgalactosaminyltransferase (GCNT1). Increased sialylation by ST6GAL1 prevents galectin-1 binding and reduces cell death (Amano et al. 2003), while GCNT1 creates viable ligands for galectin-1 and leads to T-cell death (Nguyen et al. 2001). These observations support the concept of a functional role for glycans in cell growth regulation through their expression on target glycoproteins.

Very few inhibitors for O-glycan processing have been reported. Inhibitors specific for the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (GALNT1-14, EC 2.4.1.41) based on the nucleotide-sugar donor UDP-GalNAc have been developed (Winans and Bertozzi 2002), and the glycoside 1-benzyl-2-acetamido-2-deoxy-α-O-d-galactopyranoside (α-OBn GalNAc), a structural analog of GalNAc-alpha-1,-O-serine/threonine, acts as a competitive inhibitor of O-glycan chain extension (Huet et al. 2003). α-OBn GalNAc acts as an acceptor for glycosyltransferases which extend protein-bound GalNAc chains and leads to the formation of benzyloligosaccharides which individually function as competitive inhibitors throughout the O-glycosylation biosynthetic pathway (Zanetta et al. 2000; Huet et al. 2003). These nonphysiological inhibitors are also designed to generate aryl-glycan structures which might be expected to have an impact on cell growth and/or survival. Activity of α-OBn GalNAc and its analogs were therefore screened in these colorectal cancer cell lines at 0.5 mM and tested in the human colorectal cancer cell lines (Corfield et al. 2001; Linden et al. 2008).}

Our studies provide direct evidence that O-glycosylation pathways play a role in the regulation of cell growth through apoptosis and proliferation pathways. A series of small molecular weight analogs of the GalNAc-α-1-O-serine/threonine structure based on 1-benzyl-2-acetamido-2-deoxy-α-O-d-galactopyranoside have been synthesized and tested in the human colorectal cancer cell lines PC/AA/C1/SB10C and HCA7/C29. Three inhibitors, 1-benzyl-2-acetamido-2-deoxy-α-O-d-galactopyranoside, and the corresponding 2-azido- and C-glycoside analogs were screened in these colorectal cancer cell lines at 0.5 mM and showed induction of apoptosis and downregulation of proliferation. Treatment of both cell lines with inhibitors led to changes in glycosylation detected with peanut lectin. The inhibition of glycosyltransferase activity in cell homogenates from human colorectal mucosal cells and cultured cell lines could be shown. The competitive action of the inhibitors resulted in the intracellular formation of 28 aryl-glycan products which were identified by MALDI and electrospray mass spectroscopy. The structures showed a differential pattern for each of the inhibitors in both cell lines. Gene array analysis of the glycogenes illustrated a pattern of glycosyltransferases that matched the glycan structures found in glycoproteins and aryl-glycans formed in the PC/AA/C1/SB10C cells; however, there was no action of the three inhibitors on glycogen transcript levels. The inhibitors act at both intermediary metabolic and genomic levels, resulting in altered protein glycosylation and aryl-glycan formation. These events may play a part in growth arrest.

Keywords: apoptosis/aryl-glycans/benzyl-O-GalNAc/growth inhibition/O-glycans

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### Table I. Inhibitors of O-glycosylation, analogs of benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>No</th>
<th>Abbreviation</th>
<th>Structure</th>
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</thead>
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<tr>
<td>Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside</td>
<td>1</td>
<td>α-OBn GalNAc</td>
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</tr>
<tr>
<td>2-Phenylethyl 2-acetamido-2-deoxy-α-D-galactopyranoside</td>
<td>2</td>
<td>α-OPE GalNAc</td>
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</tr>
<tr>
<td>Cyclohexyl 2-acetamido-2-deoxy-α-D-galactopyranoside</td>
<td>3</td>
<td>α-OCyHex GalNAc</td>
<td><img src="#" alt="Structure" /></td>
</tr>
<tr>
<td>Benzyl 2-azido-2-deoxy-α-D-galactopyranoside</td>
<td>4</td>
<td>α-OBn GalN3</td>
<td><img src="#" alt="Structure" /></td>
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<tr>
<td>2-Phenylethyl 2-azido-2-deoxy-α-D-galactopyranoside</td>
<td>5</td>
<td>α-OPE GalN3</td>
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<td>Cyclohexyl 2-azido-2-deoxy-α-D-galactopyranoside</td>
<td>6</td>
<td>α-OCyHex GalN3</td>
<td><img src="#" alt="Structure" /></td>
</tr>
<tr>
<td>2-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-1-phenylethane</td>
<td>7</td>
<td>α-CBn GalNAc</td>
<td><img src="#" alt="Structure" /></td>
</tr>
<tr>
<td>3-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-1-phenylpropane</td>
<td>8</td>
<td>α-CPP GalNAc</td>
<td><img src="#" alt="Structure" /></td>
</tr>
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</table>

End products accumulate in intracytoplasmic vesicles leading to a storage-type phenomenon (Hennebicq-Reig et al. 1998; Huet et al. 2003; Ulloa and Real 2003). In the human colorectal goblet cell line HT29 MTX, both constitutive and secretagogue sensitive pathways of mucin secretion are blocked after exposure to α-OBn GalNAc (Hennebicq-Reig et al. 1998; Huet et al. 2003) in addition to a specific decrease in apical cell membrane glycoproteins (Ulloa et al. 2000; Delacour et al. 2003; Huet et al. 2003). A closer examination of the action of α-OBn GalNAc in different cell lines revealed a relationship with the complement of glycosyltransferases expressed in each cell line (Gouyer et al. 2001). The inhibition of apical targeting and cellular events linked with the storage of the α-OBn GalNAc metabolites occur as independent events (Leteurtre et al. 2003). In addition, treatment of HeLa cells with 5 mM α-OBn GalNAc has been shown to induce an increase in apoptosis (Li et al. 2007).

Disaccharide decoys have been designed to inhibit the expression of the cancer antigen and selectin ligand sialyl-Lewis A (sialyl-Le^a^) (Brown et al. 2003; Fuster et al. 2003) and show that specific glycan inhibitors can be used to target defined carbohydrate antigens in biological processes.

The design of the inhibitors 1–8 (Table I), used in this study, was based on the structural features of α-OBn GalNAc (1). Compound 2 presents an extra carbon between the anomic oxygen and the phenyl group, while 3 contains a cyclohexane ring instead of the aromatic substituent. Compounds 4, 5, and 6 feature an azide functionality at C2, instead of the N-acetyl, which introduces a change not only in the size of the substituent
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Fig. 1. O-Glycosylation inhibitors do not inhibit N-glycan and O-GlcNAc biosynthesis. (A) Western blotting of PC/AA/C1/SB10C and HCA7/C29 whole cell lysates, on a 10% gel, stained with ConA lectin to detect proteins with N-glycosylation. The cells were either untreated or treated with 0.5 mM of 1, 4, and 7 for 4 days. Equal loading was verified by probing for α-tubulin. (B) Western blotting of PC/AA/C1/SB10C and HCA7/C29 whole cell lysates, on a 10% gel, stained with the anti-O-GlcNAc antibody CTD 110.6 which recognizes proteins substituted with α-O-GlcNAc on serine or threonine residues. A main band at approx. 70 kDa was unaffected by treatment with the inhibitors. Equal loading was verified, by probing for α-tubulin.

Results

α-OBnGalNAc analogs inhibit O-GalNAc-type glycosylation

The library of α-OBn GalNAc analogs (Table I) was designed to detect new inhibitors specific for O-GalNAc glycosylation and independent of the other major glycosylation pathways. Previous reports have indicated that some of the aryl-glycans generated may mimic structures shared by both O-GalNAc and N-glycan pathways (Zanetta et al. 2000; Huet et al. 2003). The inhibitors were tested for their action on the biosynthesis of N-glycans using the lectin ConA as a general screen for these glycoproteins and were unable to detect any action at 0.5 mM for 4 days in PC/AA/C1/SB10C and HCA7/C29 cells with either SDS–PAGE Western blots (Figure 1A) or confocal microscopy in PC/AA/C1/SB10C cells (data not shown). In addition, the presence of O-N-acetyl-D-glucosamine (O-GlcNAc) glycosylation, widely found in cytoplasmic and nuclear proteins, was tested with a specific antibody, CTD110.6 (Comer et al. 2001) in PC/AA/C1/SB10C and HCA7/C29 cells. Western blotting of cell lysates revealed a major band at approximately 70 kDa, in both cell lines, which was unaffected in the presence of the O-glycan inhibitors α-OBn GalNAc (1), benzyl...
2-azido-2-deoxy-α-D-galactopyranoside (α-OBn GalN3) (4) and α-Cbn GalNAC (7) (Figure 1B). Confocal microscopy also showed no change in staining with CTD110.6 in the presence of the same three inhibitors in PC/AA/C1/SB10C cells (data not shown). These results show that the inhibitors have limited action on α-N-glycan and O-GlcNAc biosynthesis.

α-OBn GalNAc analogs inhibit glycosyltransferases responsible for O-GalNAc-type glycosylation

In order to demonstrate the inhibitory action of the inhibitors, the activity of core 1 synthase, glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase 1 (coded by the C1GALT1 gene) and N-acetylgalactosaminidase 2,6-sialyltransferase (coded by the ST6GALT1 gene), two enzymes acting on the initial O-glycan and GalNAc biosynthesis.

ST6 GalNAc transferase

<table>
<thead>
<tr>
<th>Enzymes inhibitors</th>
<th>Colon res margin</th>
<th>PC/AA/C1/SB10C</th>
<th>HCA7/C29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core 1 synthase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1554.5 ± 191.1</td>
<td>480.0 ± 25.4</td>
<td>258.5 ± 33.2</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>1267.5 ± 113.8</td>
<td>428.5 ± 17.7</td>
<td>226.5 ± 4.9</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>1166.0 ± 42.4</td>
<td>302.5 ± 10.4**</td>
<td>210.0 ± 15.5</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>1116.0 ± 203.6*</td>
<td>266.5 ± 16.3***</td>
<td>200.5 ± 12.0*</td>
</tr>
<tr>
<td>α-OBn GalNAc (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>1347.5 ± 85.6</td>
<td>419.0 ± 4.2</td>
<td>247.0 ± 5.0</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>1327.0 ± 227.7</td>
<td>384.5 ± 21.9**</td>
<td>232.5 ± 17.7</td>
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<tr>
<td>1.0 mM</td>
<td>1249.0 ± 56.6</td>
<td>392.0 ± 7.1**</td>
<td>247.0 ± 18.4</td>
</tr>
<tr>
<td>α-Cbn GalNAc (7)</td>
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</tr>
<tr>
<td>0.1 mM</td>
<td>1258.0 ± 55.1</td>
<td>414.5 ± 20.5*</td>
<td>256.0 ± 5.3</td>
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<tr>
<td>0.5 mM</td>
<td>1240.0 ± 7.1</td>
<td>367.0 ± 39.6**</td>
<td>206.0 ± 11.3*</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>1146.5 ± 61.5*</td>
<td>368.0 ± 1.4***</td>
<td>233.5 ± 9.2*</td>
</tr>
</tbody>
</table>

Table II. Action of inhibitors on glycosyltransferase activity in colon cell homogenates

The action of inhibitors 1, 4, and 7 was tested in homogenates of human resection margin mucosal cells, PC/AA/C1/SB10C and HCA7 cells assayed for core translation and sialyl α2,6, GalNAc transferase using mucin substrates as described in Material and methods. Data represent means of two independent experiments ± SD. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001 versus control by Dunnett’s t-test.

In order to demonstrate the inhibitory action of the inhibitors, the activity of core 1 synthase, glycoprotein-N-acetylglucosamine 3-β-galactosyltransferase 1 (coded by the C1GALT1 gene) and N-acetylgalactosaminidase 2,6-sialyltransferase (coded by the ST6GALT1 gene), two enzymes acting on the initial O-glycan and GalNAc biosynthesis. The library of inhibitors, 1, 73 downregulated, and 14,720 nonessential and excluded from the dataset. Analysis of the remaining 14,720 expressed transcripts showed significant up-regulation of a short-term response after 14 h exposure (Delacour et al. 2003). We investigated a series of colorectal cell lines to assess the expression of the regulated genes in the PC/AA/C1/SB10C cell line. This was achieved utilizing the Affymetrix Human Genechip U133A 2.0, which contains 22,283 transcripts. Two independent experiments with the PC/AA/C1/SB10C cells cultured under control conditions as well as two independent experiments of the PC/AA/C1/SB10C cells after exposure to 0.5 mM of each of the different inhibitors 1, 4, or 7 for 4 days were performed and showed correlation coefficients >0.954 (data not shown). Transcripts that were found to be absent in all analyzed experiments were considered as nonessential and excluded from the dataset. Analysis of the remaining 14,720 expressed transcripts showed significant up-regulation of the regulated genes.

**Treatment with O-glycan inhibitors 1–8 results in growth inhibition**

Earlier studies with HT29 MTX cells showed decreased cell numbers after permanent exposure to 1 over 14 days (Hemnebicq-Reig et al. 1998; Hu et al. 2003) and evidence of a short-term response after 14 h exposure (Delacour et al. 2003). We investigated a series of colorectal cell lines to assess the expression of the regulated genes in the PC/AA/C1/SB10C cell line. This was achieved utilizing the Affymetrix Human Genechip U133A 2.0, which contains 22,283 transcripts. Two independent experiments with the PC/AA/C1/SB10C cells cultured under control conditions as well as two independent experiments of the PC/AA/C1/SB10C cells after exposure to 0.5 mM of each of the different inhibitors 1, 4, or 7 for 4 days were performed and showed correlation coefficients >0.954 (data not shown). Transcripts that were found to be absent in all analyzed experiments were considered as nonessential and excluded from the dataset. Analysis of the remaining 14,720 expressed transcripts showed significant up-regulation of the regulated genes. Two of the 14,720 expressed transcripts were chosen to examine the nature of inhibition based on their relative sensitivity to the inhibitors. Since all of the compounds tested in these cell lines led to growth inhibition, we chose representative from each of the three main groups of analogs, the parent compound, α-OBn GalNAc (1), an azide substituted O-glycoside, α-OBn GalN3 (4), and a C-glycoside, α-Cbn GalNAc (7) for further detailed studies.

**Gene array analysis of gene expression changes in PC/AA/C1/SB10C cells treated with inhibitors 1, 4, and 7**

Having shown that the inhibitors led to growth inhibition, we decided to evaluate the action of inhibitors 1, 4, and 7 on gene expression in the PC/AA/C1/SB10C cell line. This was achieved utilizing the Affymetrix Human Genechip U133A 2.0, which contains 22,283 transcripts. Two independent experiments with the PC/AA/C1/SB10C cells cultured under control conditions as well as two independent experiments of the PC/AA/C1/SB10C cells after exposure to 0.5 mM of each of the different inhibitors 1, 4, or 7 for 4 days were performed and showed expression level change for each inhibitor. Figure 1A shows increased expression of the regulated genes.

**O-Glycan inhibitors block cell growth**
**Fig. 2.** O-Glycosylation inhibitors induce growth arrest. (A) Treatment of PC/AA/C1/SB10C cells with 0.2, 0.5, and 2 mM of the α-OBn GalNAc (1) for 4 days resulted in a decreased adherent cell yield with a corresponding increase in cell shedding. Adherent cells are represented as a percentage of control. Data represent means of three independent experiments each conducted in triplicate ± SD. NS: not significant, ***P < 0.001 versus control by Dunnett’s t-test. (B) The percentage of adherent cells after incubation with 0.5 mM of inhibitors 1–8 (see Table I) for 4 days is shown relative to the control with no inhibitor (100%). Representative values from one flask per experiment of at least two experiments.

**Up-regulated**

**α-OBn GalNAc (1)**

**Down-regulated**

**α-OBn GalN3 (4)**

**α-CBn GalNAc (7)**

**Fig. 3.** Regulated genes in PC/AA/C1/SB/10C cells after exposure to O-glycan inhibitors. The action of α-OBn GalNAc (1), α-OBn GalN3 (4), and α-CBn GalNAc (7) inhibitors on gene expression examined in PC/AA/C1/SB/10C cells using the Affymetrix Human Genechip U133A 2.0. Annotation was performed with the help of NCBI and Swiss Prot databases. Analysis of 14,720 expressed transcripts showed significant (P < 0.05) up- and downregulation for each inhibitor. The data can be accessed at the GEO repository (accession number GSE12422).
genes, especially those in the upregulated group. α-OBn GalN3 (4) led to a large downregulation of proliferation genes, with upregulation of signal transduction, protein degradation, and transcription genes. The C-glycoside (7) mainly upregulated transcription, adhesion, and signal transduction genes, while proliferation, transcription, and metabolism genes were the main groups downregulated. Although there are differences in patterns between the inhibitors, the major groups of genes affected are in the transcription, proliferation, and signal transduction groups (Figure 3). The gene expression data are available at the GEO repository http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12422, accession number GSE12422.

O-Glycosylation inhibitors induce apoptosis in PC/AA/C1/SB10C and HCA7/C29 cells

To assess the nature of cell growth inhibition in PC/AA/C1/SB10C and HCA7/C29 cells, we determined whether the induction of apoptosis played any role in this process. Culture with 0.5 mM of 1, 4, or 7 for 4 days led to a reduction of adherent cells and an increase in the proportion of cell shedding for PC/AA/C1/SB10C and HCA7/C29 cells that was statistically significant (Figure 4A). The increase in shed cells was more pronounced with α-OBn GalN3 and α-CBN GalNAc compared to α-OBn GalNAc versus control.

Due to the increase in shed cell numbers inhibitor-treated cell samples were tested for apoptotic morphology by AOEB staining. All shed cells from control and treated samples in both cell lines showed membrane blebbing, condensation and segregation of nuclear chromatin, and cellular shrinkage; all are the characteristics of apoptotic cells as described previously (Figure 4C, lanes Co, 1, 4 and 7 (Hague et al. 1993)). Moreover, apoptosis was further confirmed by the cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). In untreated adherent cells, PARP was predominantly uncleaved (Figure 4C, lane Ad), while in shed cells from untreated (control) and treated samples, PARP was cleaved, as expected for apoptotic cells as described previously (Figure 4C, lanes Co, 1, 4 and 7 (Hague et al. 1993)). Moreover, treatment with all inhibitors induced a modest but statistically significant increase in the executioner caspase 3 and 7 activity (Figure 4D). The increased apoptosis observed here is in agreement with earlier studies with HeLa cells (Li et al. 2007).

Action of O-glycosylation inhibitors on proliferation in PC/AA/C1/SB10C and HCA7/C29 cells

An influence of the inhibitors on proliferation gene expression could be shown in PC/AA/C1/SB10C cells (Figure 5A) and most of these genes were significantly downregulated. This effect was most apparent with the azide 4 and the C-glycoside 7, while 1 was less effective (Figure 5A).

In order to verify the gene array data at the protein level, we chose to investigate the expression of Ki67 a well-known proliferation marker (McCormick et al. 1993). Treatment with all inhibitors reduced staining for Ki67 in PC/AA/C1/SB10C cells (Figure 5B and C) and HCA7/C29 cells (data not shown). This is in good agreement with the gene expression results, where MKI67, the gene coding for the Ki67 protein, is downregulated for all inhibitors, but with only the azide 4 showing a factor of 2 (Figure 5A). Significantly, more cells were negative in the treated cell populations for both cell lines especially after treatment with 4 and 7, but to a lesser degree with 1 (Figure 5B and C for PC/AA/C1/SB10C cells).

O-Glycosylation inhibitors lead to truncated cellular glycans and increased terminal β-galactose

Previous indication of cell surface glycoconjugate recognition in pathways regulating apoptosis and proliferation (Gabius 2006) prompted us to identify the nature of cellular glycosylation changes resulting from exposure to the O-glycosinhibitors. The competitive inhibition of O-glycosylation by 1 and its analogs leads to a general disruption of cellular glycan structures. Many truncated glycans terminated with β-galactose residues are found in the membrane proteins (Zanetta et al. 2000; Huet et al. 2003). The mature sialylated and modified glycans can be detected using lectins that recognize the terminal monosaccharides. Peanut lectin (PNA) recognizes the disaccharide Galβ1-3GalNAc-R (core 1 or Thomsen–Friedenreich (TF) antigen) and has a general affinity for glycans with terminal βGal residues (Gouyer et al. 2001). Accordingly, we observed a marked upregulation of PNA staining in PC/AA/C1/SB10C cells treated with all inhibitors as shown in Figure 6 for 1, 4, and 7. Similar results were found with HCA7/C29 cells (data not shown).

The sialylation pattern varied with each cell line used. α2-3-linked sialic acids were detected with Maackia amurensis II lectin (MALII). PC/AA/C1/SB10C cells were positive for the lectin, but showed no influence of any inhibitor. No staining was observed with HCA7/C29 cells (data not shown). The detection of α2-6-linked sialic acid with Sambucus nigra lectin (SNA) was positive for both PC/AA/C1/SB10C and HCA7/C29 cells but also revealed no change on exposure to inhibitors 1, 4, and 7 (data not shown). The fucose-binding lectin Ulex europaeus I which recognizes Fucα1-2Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc-R (core 1) and its analogs led to positive staining in both PC/AA/C1/SB10C and HCA7/C29 cells, but exposure to inhibitors 1, 4, and 7 showed no change (data not shown).

These lectin analyses demonstrate that the modifications in glycosylation relate to glycan truncation and increased β-galactose chain termination and occur at the cell surface and other subcellular locations after exposure to the inhibitors. These changes represent target structures leading to the modulation of cell growth pathways and correlate well with the analysis of the aryl-glycans formed in these cells (see below and Table IV) and the gene array data for the glycosyltransferases expressed in the PC/AA/C1/SB10C cell line (see below).

O-Glycosylation inhibitors lead to the synthesis of aryl-glycans

The competitive action of the inhibitors is believed to arise due to the intracellular formation of products through the action
of cellular glycosyltransferases on the inhibitors. In this way, a series of aryl-glycan structures are synthesized which act as step-specific acceptors for the glycosyltransferases and compete out the natural cellular substrates. As a result, cellular glycoconjugates have depleted abnormal glycan chains as shown above (Figure 6). Identification of the aryl-glycan products, therefore, will give an indication of the individual glycosylation steps that may be inhibited and provide a series of glycan structures with defined inhibitory activity. Previous studies have shown that aryl-glycan products are formed and accumulate within cells exposed to α-OBn GalNAc (Zanetta et al. 2000). We chose to examine PC/AA/C1/SB10C and HCA7/C29 cells after exposure to 0.5 mM of α-OBn GalNAc, α-OBn GalN3, and α-CBn GalNAc for 4 days and harvesting of the products by Folch extraction of the cells. The analysis of the chloroform/methanol extracts by matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry yielded the 28 products shown in Table IV. PC/AA/C1/SB10C cells showed the largest number of products, 24 for α-OBn GalNAc (1), while α-CBn GalNAc (7) yielded 17 and α-OBn GalN3 (4) only 8. HCA7/C29 cells produced fewer.
O-Glycan inhibitors block cell growth

**Fig. 5.** Downregulation of proliferation-related genes in colorectal cancer cell lines. (A) Treatment of PC/AA/C1/SB10C cells with 0.5 mM of 1, 4, and 7 for 4 days resulted mainly in the downregulation of expression of a number of proliferation-related genes by a factor of 2 as detected by the Affymetrix Human Genechip U133A 2.0. This list contains the statistically significant affected genes from two independent experiments. The data can be accessed at the GEO repository (accession number GSE12422). (B) PC/AA/C1/SB10C cells were grown on coverslips and treated with 0.5 mM of 1, 4, and 7 for 4 days. The slides were stained with DAPI and a Ki67 MAb, followed by an Alexa Fluor 488-conjugated secondary antibody and 1000 cells from one slide per treatment were counted under a fluorescence microscope. Treatment with all inhibitors especially 4 and 7 resulted in the downregulation of Ki67 expression. The experiment was repeated three times and the numbers of Ki67-positive cells were plotted as a percentage of the control. All Ki67-positive cells were counted irrespective of differences in the intensity of Ki67-positive per cell signal. Negative untreated controls with only Alexa Fluor 488-conjugated antibody did not reveal any green staining (data not shown). Data represent means of three independent experiments ± SD. **P < 0.01, ***P < 0.001 versus control by Dunnett’s t-test. (C) Typical pictures of Ki67 expression with or without treatment were obtained at a 20 × magnification under an immunofluorescence microscope.

aryl-glycans with 12 identified for α-OBn GalNAc, 5 for α-OBn GalN1, and none for α-CBn GalNAc.

The aryl-glycan structures identified (Table IV) concur with similar experiments reported with HT29 G- and HT29 MTX cells (Zanetta et al. 2000; Gouyer et al. 2001; Huet et al. 2003) and in agreement with known glycosylation pathways reported in the literature (Schachter and Brockhausen 1992; Zanetta et al. 2000).

Sequencing of oligosaccharides was acquired in both positive- and negative-ion modes to obtain complete elucidation of the structure for each mucin oligosaccharide. Positive-ion mode is useful for the determination of the core-type oligosaccharide and the position of fucose, whereas negative-ion mode produces characteristic cross-ring cleavages necessary for differentiation between isolomers and identification of NeuAc (N-acetylneuraminate) substitution. A series of diagnostic ions were used to determine the linkages between monosaccharides (Robbe et al. 2006). The ions at m/z 306 and 513 are specific for NeuAcα2-6 linked to GalNAc whereas the ions at m/z 408 or 611 are characteristic of NeuAcα2-3 linked to a Gal residue. A specific 0.3A1 cleavage with the concomitant loss of water from a GlcNAc substituted on C-4 allowed to differentiate between type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) chains. Some characteristic ions also allowed assigning unambiguously the presence of certain terminal structures such as the Lewis determinants. However, in complex oligosaccharides, diagnostic ions are sometimes of lower intensity, rendering the complete elucidation of the structure difficult or impossible. Designation of oligosaccharide sequence from the MS data is shown in Table IV.

The MS data identify structures corresponding to core 1 (Galβ1-3GalNAc-), core 2 (Galβ1-3GlcNAcβ1-6)GlcNAc-), core 2 with a Galβ1-3Galβ1-3 arm (Galβ1-3GlcNAcβ1-6)GlcNAc-), core 3 (GlcNAcβ1-3GlcNAcβ1-6)GlcNAc-), and core 4 (GlcNAcβ1-3GlcNAcβ1-6)GlcNAc-). Each of these shows fucosylated and sialylated intermediates but contains no sulfated glycans.

Core 1 (1) structures include sialylated (5 and 28) and digalactosyl (2 and 4) products, while most of the aryl-glycans identified were based on the core 2 structure (3). The core 2 pathway contains structures with a Galβ1-3Galβ1-3 arm (7, 9, 10, 12, 15, 18, 19, and 21), a disaccharide not found in established biosynthetic pathways, but corroborated by NMR analysis in previous studies (Zanetta et al. 2000). Other glycans based on
Table IV. Proposed glycan structures from nano ESI Q-TOF data of inhibitor glycans extracted from cells cultured with inhibitors

<table>
<thead>
<tr>
<th>Product no.</th>
<th>Proposed structure</th>
<th>PC/AA/C1/SB10C</th>
<th>HCA7/C29</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>Galβ1-3GalNAc-aryl (1)</td>
<td>496</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Galβ1-3GalNAc-aryl (1)</td>
<td>658</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>a. Galβ1-3GalNAc-aryl (1)</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>b. Galβ1-3GalNAc-aryl (1)</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>c. Galβ1-3GalNAc-aryl (1)</td>
<td>-</td>
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<td></td>
<td>d. Galβ1-3GalNAc-aryl (1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Galβ1-3(Fucα1-2)Galβ1-3GalNAc-aryl (1)</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Neu5Ac2-3Galβ1-3GalNAc-aryl (1)</td>
<td>891</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>a. (Fucα1-2)Galβ1-3GalNAc-aryl (1)</td>
<td>845</td>
<td>829</td>
</tr>
<tr>
<td></td>
<td>b. Galβ1-3(Fucα1-2)Galβ1-3GalNAc-aryl (1)</td>
<td>-</td>
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<tr>
<td></td>
<td>c. (Fucα1-2)Galβ1-4GalNAc-aryl (1)</td>
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<td></td>
<td>d. (Fucα1-2)Galβ1-3GalNAc-aryl (1)</td>
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<td>8</td>
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<td></td>
<td>b. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>c. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>d. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>e. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>f. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>g. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>h. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>i. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>j. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>k. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>l. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>m. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>n. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
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<td>p. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>q. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>r. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>s. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>t. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>u. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>v. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>w. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td></td>
<td>x. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>y. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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<td>z. Galβ1-3Galβ1-3GalNAc-aryl (1)</td>
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</table>

Core 1 & 2 (1), Core 3 & 4 (3) and Core 6 (6) related structures are indicated by the numbers in parentheses after each structure. GalNAc-aryl indicates the structure relative to each of the inhibitors 1, 4 and 7 as indicated in Table I.
core 2 account for the extension of both Galβ1-3 and GlcNAcβ1-6 arms, with intermediate fusocysylation or sialylation (6–22, 24–27). Aryl-glycans also include core 3 and 4 structures (e.g., 11, 16, 20, 22, 23, and 24).

The products formed for both PC/AA/C1/SB10C and HCA7/ C29 cells show differential patterns for each of the three inhibitors in each cell line (Table IV). Fewer products were detected in the HCA7/C29 cell line compared to PC/AA/C1/ SB10C. The absence of any products for the C-glycoside 7 is particularly striking in this cell line. Taken together, these data show differential utilization of the three inhibitors by PC/AA/C1/ SB10C and HCA7/C29 cells under the same conditions and reflect the utilization of the glycome expressed in each cell line. Further support for the arylglycan structures can be implemented from the Affymetrix Human Genechip U133A 2.0 gene array database for the PC/AA/C1/SB10C cells. This is detailed below.

Glycogenes account for PC/AA/C1/SB10C glycosylation patterns but are not influenced by O-glycosylation inhibitors

The inhibition of cell growth by the inhibitors implicates functional changes with key proteins mediating these processes. The examination of the gene coding for the enzymes synthesising the glycans may indicate which structures can be formed and corroborate the aryl-glycans identified by mass spectrometric analysis in Table IV.

The effect of the inhibitors in the PC/AA/C1/SB10C cell line was monitored against the database of glycogenes detailed by the Consortium for Functional Glycomics (URL, http://www.functionalglycomics.org/glycomics/publicdata/microarray.jsp; Gene list, http://www.scripps.edu/researchservices/dna_garry/lyco_genelist.xls). None of the genes expressed in the PC/AA/C1/SB10C cells and listed in supplementary Table I show a significant response to the three inhibitors tested. These data can be accessed at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12422, accession number GSE12422).

Thus, a direct action for the inhibitors at the genomic level is not observed for glycogenes.

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Thus, a direct action for the inhibitors at the genomic level is not observed for glycogenes.
The sialyltransferase genes identified are also responsible for the synthesis of the nonfucosylated Sd\(^4\) antigen from both type 1 and type 2 chain precursors (represented by 7, 8, and 16). This occurs under the influence of the Sd\(^4\) enzyme β4GalNAcT coded for by B4GALNT1 and present in the PC/AA/C1/SB10C cells (supplementary Table I). Both type 1 and type 2 Sd\(^4\) structures are found in mucins isolated from the PC/AA/C1/SB10C cell line confirming that α2-3-sialylated N-acetyllactosamine precursors are formed. However, no Sd\(^4\) motifs are found in the aryl-glycans. Studies on the molecular biological basis for Sd\(^4\) and sialyl-Le\(^x\) expression have indicated that a competition between B4GALNT and α1-3/4 fucosyltransferases exists and that this may govern the appearance of ectopic sialyl-Le\(^x\) on glycoproteins in colon cancer (Malagolini et al. 2007).

The expression of a β-galactoside α2-6-sialyltransferase, ST6GAL1, is consistent with the SNA lectin binding reported above. However, identification of α2-6 sialic acids linked to galactose in the aryl-glycans was not made. This enzyme probably acts on N-glycans in this cell line. Furthermore, the expression of ST8SIA genes most likely correlates with N-glycan synthesis as no di- or polysialyl structures were detected in the aryl-glycans reported here or in mucins from the PC/AA cell lines (Robbe Masselot et al., in preparation).

The aryl glycan products formed in both PC/AA/C1/SB10C and HCA7/C29 cell lines carried no sulfate. Sulfation of O-glycans may arise due to the action of a series of glycosulfotransferases detected in PC/AA/C1/SB10C cells (supplementary Table I). Most of these are involved in GAG biosynthesis, but GAL3ST1 and GAL3ST4 may account for the presence of fucosylated aryl-glycans. Studies on the molecular biological basis for these cells and implicates the aryl-glycans as potential inhibitors of PC/AA/C1/SB10C cell growth.

Discussion

The O-glycan inhibitor α-OBn GalNAc has been widely used in cell culture studies; as O-glycosylation is implicated in galectin-based membrane trafficking, cell signalling (Fuster et al. 2003; Huet et al. 2003; Ulloa and Real 2003; Gabius 2006), and apoptosis (Gabius 2006), it was attractive to focus attention on a potential role in the regulation of cell growth. In order to improve our understanding of the O-glycan pathways mediating the cell growth of human colorectal cell lines, used in our previous glycobiological studies, we have designed new analogs of α-OBn GalNAc which all lead to growth inhibition, the induction of apoptosis, downregulation of proliferation gene expression, and modified O-glycosylation. We show for the first time that such manipulation results in the generation of aryl-glycan products and that this inhibition is a result of action at the intermediary metabolic and genomic levels.

The testing of compounds 1, 4, and 7 for their specificity with regard to glycosylation confirmed that they are active on the O-GalNAc glycosylation pathways. The N-acetylhexasamines, GalNAc and its epimer GlcNAc are monosaccharides, which form the glycosidic linkage in O-glycan, N-glycan, and O-GalNAc glyc–protein linkages. The direct testing of the N-glycan and O-GlcNAc linkages in the colorectal cell lines gave no indication for inhibitory action on these posttranslational glycosylation events (Figure 1). Inhibition of glycosyltransferase activity with mucin substrates could be shown for compounds 1, 4, and 7 in fresh colonic mucosal cells, reaching significance with \(P < 0.05\) at 1 mM for 1 and 7, for PC/AA/C1/SB10C cells with \(P < 0.001\) at 1 mM for all inhibitors, and HCA7/C29 cells with \(P < 0.05\) at 1 mM for 1 and 7.

In the colorectal cell lines tested here, a standard culture time of 4 days was found to be sufficient to assess all of the compounds for growth arrest, with the majority showing significant inhibition at 0.5 mM. Screening showed that there was a selective inhibition pattern for the library of inhibitors (Figure 2). The susceptibility of cell lines to α-OBn GalNAc (1) inhibition is known to vary (Huet et al. 2003) and is thought to reflect the glycosyltransferase complement present (Brown et al. 2003; Huet et al. 2003). This observation does not assess the relative cellular uptake of the inhibitor nor does it account for the potency of inhibition once it is inside the cells (Brown et al. 2003). Evidence for the formation of inhibitor–metabolites provides direct proof of uptake, but the proportion of each inhibitor transported into cells is not known. The cell-specific response to each inhibitor is generated through a combination of uptake dynamics, the cellular complement of glycosyltransferases and their sensitivity to inhibition on the O-glycan synthetic pathways, and selective blocking of glycosylation for key glycoproteins required for normal cell growth.

All O-glycan inhibitors tested here induce apoptosis in the human epithelial cell lines tested. Apoptosis is well known as a regulator of cell growth (Watson 2004) and its characteristic patterns are confirmed (Figure 4) through AOEB staining, PARP cleavage, and activation of executioner caspases 3 and
Development and synthesis of new α-OBn GalNAc analogs were selected as a target because these inhibitors act at multiple, step-specific sites on the O-glycosylation pathways. Further support for a role for O-glycans in apoptosis has come through studies of MUC1 which attenuates oxidative stress-induced apoptosis (Yin et al. 2003) and the intrinsic pathway (Ren et al. 2004) and is a target for galectin-3 binding through its core 1 glycans (Yu et al. 2007).

The action of the inhibitors at the metabolic level is demonstrated by the inhibition of glycosyltransferase activity and with the isolation of a distinct series of aryly-glycans for each inhibitor (Table IV). Thus, chemical manipulation of the α-OBn GalNAc structure yields new information on the sites of inhibition throughout the O-glycosylation pathways and expands previous data with HT29 cell lines for α-OBn GalNAc (1) alone (Zanetta et al. 2000). The use of N-acetyllactosamine disaccharide naphthalenemethanol primers to inhibit sialyl-Lea formation also generated a series of extended products, characteristic for each inhibitor, when tested in U937 cells (Sarkar et al. 2000; Fuster and Esko 2005). The products identified for the PC/AA/C1/SB10C and HCA7/C29 (Table IV) lines map directly onto O-glycan metabolic pathways for core 1-4 synthesis and extension. The individual inhibitors generate a selective pattern of aryly-glycans (Table IV) and it can be proposed that the initial products formed inhibit key glycosyltransferases and block the further extension of the glycans.

Fewer aryly-glycans were found in the HCA7/C29 cells, but those found correspond to the PC/AA/C1/SB10C products without the N-acetyllactosamine extensions seen on the core 2 and 4 structures. The most striking result was the absence of any products for the C-glycoside 7. These results could be explained due to a smaller range of glycosyltransferases in HCA7/C29 compared with PC/AA/C1/SB10C cells, although it is more likely that less inhibitor is taken up by the HCA7/C29 and the sensitivity of the MS analysis leads to fewer products being detected. The intracellular concentrations of inhibitor required to trigger these events are likely to be lower than 0.5 mM, as administered in the cell incubations.

Several features of the aryly-glycans formed by the PC/AA/C1/SB10C and HCA7/C29 cells indicate that a selective and unique use of the glycogenes operates in each cell line. This is reflected in the presence of nonnatural structures and the exclusion of some structures that are formed in vivo with natural substrates. The glycosyltransfer reactions generating the aryly-glycans from the inhibitors result from effective competition with the natural, protein acceptors. Accordingly, nonnatural structures containing Galβ1-3Galβ1-3GalNAc-R represent major products. None of the aryly-glycans isolated are sulfated although appropriate genes are expressed (supplementary Table I), and this is apparent for the HCA7/C29 as well as the PC/AA/C1/SB10C cells.

Sialyl Leα, but not sialyl Leβ, or the Sd2 antigen is found in the aryly-glycans. The precursors for all of these three structures are α2-3-sialylated N-acetyllactosamine. The sialyltransferases govern the preference of sialyl Leα over sialyl Leβ, while competition between the α1-3/4 fucosyltransferases leads to sialyl Leα synthesis at the expense of Sd2 although the β1-4GalNAc transferase is present. The specificity of the expressed glycosyltransferases accounts for the products detected.

The depletion of the core 3 enzyme implies that the core 3 and 4 aryly-glycans identified and carried on the PC/AA/C1/SB10C mucins arise due to the action of alternative β1-3GalNAc transferases, B3GNT1, and B3GNT3. As a result, a high proportion of core 1 and 2 products are found (Table IV). The loss of the core 3 synthase has been demonstrated as a primary event in the adenoma–carcinoma sequence in the colon and also correlates with increased metastasis (Vavasseur et al. 1994; Iwai et al. 2005). The switch to core 1 and 2 structures demonstrated here may play a role in this process.

The significance of the aryly-glycan structures identified in this study lies in their role as inhibitors of individual steps on the O-glycan synthesis. The correlation of aryly-glycans structures with the known core 1-4 pathways identifies a series of specific inhibitors. The glycans in these products all contain two or more monosaccharide components and are formed within the cells. The effective use of such structures as inhibitors requires their transport into the cells, and this has been identified as a limitation (Sarkar et al. 2000). It is assumed that these compounds are taken up by passive diffusion (Sarkar et al. 2000), and studies with other glycosylation inhibitors have employed peracetylated derivatives to improve uptake (Sarkar et al. 2000; Prescher et al. 2004; Dube and Bertozzi 2005). The acetylated inhibitors are deacetylated within the cells by nonspecific esterases. Studies with peracetylated disaccharide glycoside analogs of N-acetyllactosamine also form inhibitor-specific “primed” aryly-glycans (Sarkar et al. 2000). As the disaccharide glycosides are active in the micromolar range (Sarkar et al. 2000; Fuster and Esko 2005) compared to the monosaccharide inhibitors in this study, the aryly-glycan sequences reported here are expected to show greater potency if used as inhibitors. These inhibitors act as primers and metabolic decoys in susceptible cell lines (Brown et al. 2003; Huet et al. 2003) and have been employed to diminish the expression of sialyl-Leα which plays a crucial role in inflammatory processes and metastasis (Brown et al. 2003). Finally, it is envisaged that the aryly-glycans reported here will form a basis for the design of effective inhibitors of cell growth. They represent specific glycosyltransferase inhibitors and are relevant to the investigation of defined protein–glycan interactions in health and disease. A primary target remains the identification of the key proteins, which mediate cell growth and depend on O-glycans for their biological activity.

The gene array analysis provides evidence for the action of these inhibitors at the genomic level and shows that a significant number of genes associated with apoptosis and proliferation are affected. No direct action on expressed glycogenes was found. The implications for all of the many gene changes identified remain to be assessed.

In summary, we have found that a series of O-GalNAc-based inhibitors lead to colorectal cell growth inhibition through induction of apoptosis and inhibition of proliferation gene expression. Gene array identifies the glycogene complement and allows verification of the active glycosyltransferase-mediated pathways. The aryly-glycans formed from the inhibitors mirror the pathways found with the gene array, show variation between analogs, and allow the routes of inhibition to be mapped. The aryly-glycans represent a series of potential inhibitors with greater selectivity and potency than the original compounds. The variations observed between the inhibitors vindicate the chemical approach to develop new compound to manipulate the O-glycosylation pathways. Future work will be directed at the identification of the key O-GalNAc glycan–proteins responsible for growth inhibition.
UDP-galactose and CMP-N-acetyl-d-neuraminic acid were purchased from Merck Biosciences (UK); asialo-ovine submandibular gland glycoprotein (asialo-OSM) was prepared as described before (King et al. (1994)) and contained 0.25 μmol terminal GalNAc/mg. The following radioactive materials were New England Nuclear products obtained from PerkinElmer Lifesciences, UK: UDP-U-[14C]-galactose (11.0 GBq/mmol) and CMP-N-acetyl-d-[14C]neuraminic acid (59.2 MBq/mmol).

**Human colonic cell preparation**

Human colorectal mucosal cells were prepared from resection margin tissue obtained at surgery as described before (King et al. 1994). Ethical approval and consent was obtained for this material.

**Cell culture**

HT29 and Caco2 cell lines were obtained from the ATCC (Rockville, MD), and the HCA7/C29 cell line was a gift from Dr. Susan Kirkland, Department of Histopathology, Imperial College London, UK. All cell lines were cultured at 37°C in 5% CO2 in air in T25 flasks. HT29, Caco2, and HCA7/C29 were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM (Life Technologies, Inc., Foster City, CA)) containing 10% fetal bovine serum (FBS, Life Technologies, Inc.), 2 mM glutamine, 1 μg/mL hydrocortisone sodium succinate, 100 U/mL penicillin, 100 μg/mL streptomycin, at 37°C in 5% CO2 (standard growth medium), routinely passaged using 1% trypsin (Difco, Sparks, MD)/0.1% EDTA (Analar, BDH, VWR International Ltd., Lutterworth, UK), PC/AA/C1 and PC/AA/C1/SB10C cells were cultured in a conditioned medium as previously described (Paraskeva et al. 1984; Williams, Browne, et al. 1993; Williams, Hague, et al. 1993). PC/AA/C1 is an adenoma-derived cell line. The PC/AA/C1 adenoma cell line is anchorage independent and nontumorigenic in athymic nude mice, while PC/AA/C1/SB10C is an anchorage-independent tumorigenic derivative of the PC/AA/C1 adenoma cell line (Paraskeva et al. 1984; Williams, Browne, et al. 1993; Williams, Hague, et al. 1993). Three days after seeding, cells of each cell line were incubated in the presence or absence of each of the inhibitors as given below, in either standard growth medium or conditioned medium, for 4 days. Shed cells floating in the medium were collected by centrifugation at 3000 rpm for 3 min, and adherent cells were washed in 1× PBS (phosphate-buffered saline), removed by trypsinization (0.1% trypsin, 0.1% EDTA), and collected by centrifugation at 3000 rpm for 3 min. Adherent and shed cells from each flask were counted separately, and the shed cells were represented as a percentage of the total cell yield as previously described (Diaz et al. 2000).

**Cell culture with O-glycan inhibitors**

All cells were cultured as described above in the presence of 0–2 mM of inhibitors 1–8 (Table I) for up to 4 days. Based on the results obtained with the initial screening, compounds 1, 4, and 7 were selected for a detailed examination using the techniques described below.

**Detection of apoptosis in cell culture**

We used the following methods to test whether the O-glycosylation inhibitors induced apoptosis in colon tumor cells in cell culture. We have previously shown that apoptotic cells in cultured colorectal cell lines are shed (detach) and float in the growth medium and that agents such as butyrate (Hague et al. 1993) and vitamin D (Diaz et al. 2000) induce apoptosis in colorectal tumor cell cultures. The percentage apoptosis was determined by measuring the proportion of apoptotic, shed cells as a percentage of the total cell (cells remaining attached to the tissue culture vessel plus shed, apoptotic cells) population as described in detail previously. Shed cells were confirmed as apoptotic using a combination of biochemical and morphological assays as described previously by us and others (Hague et al. 1993; Sheng et al. 1998; Diaz et al. 2000). Briefly, shed cells were confirmed as apoptotic morphologically after acridine and ethidium bromide staining (see below). Apoptosis was further confirmed biochemically by showing PARP cleavage in the floating cells and measuring caspase activity (see below).

**Acridine orange and ethidium bromide staining**

The level of apoptosis in cultured colon cells was assessed by acridine orange and ethidium bromide (AOEB) staining. Shed cells were stained with 5 μg/mL acridine orange (Sigma) and 5 μg/mL ethidium bromide (Sigma Aldrich, Poole, UK) according to Hague et al. (1993). Acridine orange penetrates living cells and fluoresces yellow/green on excitation at 450–490 nm. Ethidium bromide can only enter permeable cells and fluoresces red at the same excitation wavelength. Morphological characteristics of apoptotic cells are condensed and segregated chromatin, membrane blebbing, formation of apoptotic bodies, and cellular shrinkage (Hague et al. 1993).

**Caspase 3/7 assay**

Caspase 3 and 7 activity was measured in untreated and treated PC/AA/C1/SB10C and HCA7/C29 cells using the Caspase-Glo 3/7 assay kit (Promega, Southampton, UK). Briefly, after incubation of the cells in well plates with inhibitors, they were lysed, and cleavage of the DVED tetrapeptide by caspase action was monitored through a luciferase assay giving results for caspase 3 and 7 activity. PC/AA/C1/SB10C and HCA7/C29 cells were seeded at 20,000 cells per well in white-walled 96-well plates (BD Biosciences, Cowley, UK) and after 3 days treated with or without 0.5 mM of the inhibitors in 100 μL of medium per well for 4 days. Control blanks were run for each cell line. The Caspase-Glo 3/7 assay was carried out according to the manufacturers instructions and the luminescence measured at 620 nm. The mean of the triplicate luminescence values of the blank controls was subtracted from each of the triplicate values for every repeat, and the results were plotted as a percentage of the untreated controls.

**Western blotting and detection with antibodies and lectins**

After treatment, the growth medium was aspirated, and the cells were washed twice with 5 mL of ice-cold PBS. After all PBS was aspirated, 150 μL of ice-cold 1.5× lysis buffer (Cell
Hundred micrograms of each sample, collected and a DC Protein Assay was performed according to the manufacturers instructions (DC2 Protein Assay kit, Bio-Rad, Hemel Hempstead, UK). Hundred microliters of each sample was boiled for 5 min in 5× SDS–PAGE buffer and run on a 10% SDS–polyacrylamide gel, and then blotted onto a PVDF membrane (Millipore, UK). The membranes were blocked at room temperature (RT) for 2 h in the 4% milk buffer and incubated overnight with the appropriate antibody diluted in the 4% milk buffer, at 4°C. Uncleaved and cleaved PARP was detected using a mouse monoclonal PARP antibody (C2-10 Alexis Corporation, Bingham, UK), at 1:5000. Proteins containing O-GlcNAc were detected using a mouse monoclonal O-GlcNAc antibody (CTD110.6 Abcam, Cambridge, UK) at 1:1000 dilution. Following two 10 min washes in the 4% milk buffer, two 10 min washes in the 0.1% Tween 20 buffer, and one 10 min wash in the 4% milk buffer, the membranes were incubated at RT for 1 h with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma Aldrich, Poole, UK) at 1:1000. After one 20 min wash in the 4% milk buffer and a 10 min wash in 0.1% Tween 20, the membranes were rinsed in distilled water and the bound HRP-antibody complex was detected using enhanced chemiluminescence (ECL) (Perbio Science, Epsom, UK) and light sensitive film (MXB film, Kodak, UK). For verification of equal loading the membranes were stripped and re-probed with an α-tubulin mouse monoclonal antibody (DM1A, Sigma Aldrich, Poole, UK), at a dilution of 1:20,000 and processed as described above.

For Western blotting experiments where lectins were used, the membranes were blocked in 1× PBS containing 1% bovine serum albumin (BSA, Sigma Aldrich, Poole, UK), instead of the milk buffer, and washed in 1× PBS, 1% BSA, 0.1% Tween 20 (Sigma), instead of 0.1% Tween 20 alone, according to the protocol mentioned above. N-Glycosylated proteins, carrying the α-mannose sugar, were detected using the biotinylated lectin Concanavalin A (ConA, Vector Labs, Peterborough, UK), at 1:10,000. For secondary staining, HRP-streptavidin was used at 1:20,000.

**MALDI and Electrospray mass spectrometry for glycan structural analysis**

Determination of the sequence structure of the glycan chains present in glycoproteins or aryl-glycans was carried out using mass spectrometry and is regarded as a nonquantitative methodology (Zanetta et al. 2000; Robbe et al. 2003). For MALDI mass spectrometry, all mass spectra were obtained on a Voyager Elite (DE-STR) reflectron time-of-flight (TOF) mass spectrometer (Perceptive Biosystems Framingham, MA) fitted with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. Glycan samples were analyzed in delayed extraction mode using an accelerating voltage of 20 kV, a pulse delay time of 200 ns, and a grid voltage of 66%. Detector bias gating was used to reduce the ion current for masses below 500 Da. Between 100 and 200 scans were averaged for each mass spectrum. Glycans were co-crystallized with 2,5-dihydrobenzoic acid as a matrix (10 mg/mL 2,5-dihydrobenzoic acid in methanol/water (50/50 v/v) containing 0.1% trifluoroacetic acid supplemented with 5 mM NaCl). The “dried droplet” preparation technique was used for all measurements. Typically, 1 μL of the matrix was mixed on target with 1 μL of glycans dissolved in water and allowed to dry under an air stream. Samples were analyzed in both positive- and negative-ion modes.

For electrospray mass spectrometry (nanoESI-MS/MS), all analyses were performed on a Q-STAR Pulsar quadrupole time-of-flight (Q-q-TOF) mass spectrometer (Applied Biosystems/MDS Scieix, Toronto, Canada) fitted with a nanoelectrospray ion source (Protopan, Odense, Denmark). Oligosaccharides dissolved in water (60 pmol/μL) were acidified by the addition of an equal volume of methanol/0.1% formic acid and sprayed from gold-coated “medium length” borosilicate capillaries (Protopan). A potential of ~800 V was applied to the capillary tip and the focusing potential was set at ~100 V, with the declining potential varying between ~60 V and ~110 V. For the recording of conventional mass spectra, TOF data were acquired by accumulation of 10 MCA (multiple channel acquisition) scans over mass ranges of m/z 400–2000. In the collision-induced dissociation (CID) tandem MS analyses, multiple charged ions were fragmented using nitrogen as collision gas (5.3 × 10⁻⁵ Torr), with the collision energy varying between ~40 and ~90 eV to obtain optimal fragmentation. The CID spectra were recorded on the orthogonal TOF analyzer over a range of m/z 80–2000. Data acquisition was optimized to supply the highest possible resolution and the best signal-to-noise ratio even in the case of low abundance signals. Typically, the full width at half maximum (FWHM) was 7000 in the measured mass ranges. External calibration was performed prior to each measure using a 4 pmol/μL solution of taurocholic acid in acetonitrile/water (50:50, v/v) containing 2 mM of ammonium acetate (Robbe et al. 2003).

**Confocal and immunofluorescence microscopy**

PC/AA/C1/SB10C or HCA7/C29 cells were grown on plastic coverslips for 4 days, and the medium with the shed cells was removed. The attached cells were washed twice in PBS. Adherent cells were fixed with 2% of paraformaldehyde (PFA) for 20 min at RT and permeabilized with 0.1% (w/v) Triton X-100 (Sigma Aldrich, Poole, UK), in PBS, for 3 min. After two 5 min PBS washes, the cells were blocked in 1% (w/v) BSA for 15 min on a rocking platform and stained with 1:10,000...
of a number of biotinylated lectins or with 1/200 of a Ki67 mouse monoclonal antibody (MB5 Dako, Glostrup, Denmark) in 70 μL of PBS per slide for 1 h at room temperature (RT). After washing twice with PBS for 10 min each, 1:20,000 of a fluorescein (FITC)-streptavidin conjugate (Vector Labs, Peterborough, UK) or 1/400 of an Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) secondary antibody (Invitrogen) in 70 μL of PBS per slide was added and incubated for 1 h at RT, and the cells were washed twice with PBS. Coverslips were inverted onto glass slides with 4',6'-diamidino-2-phenylindole (DAPI [Jackson Immunoreagents, West Grove, PA]) and sealed. Laser confocal analyses were performed using a TCS-SP2 confocal imaging spectrophotometer system (Leica, Milton Keynes, UK). An oil immersion objective lens, ×63 NA 1.4, was used. Ki67-positive cells were counted under an immunofluorescence microscope.

For confocal microscopy, the biotinylated lectins (Vector Labs, Peterborough, UK), Concanavalin A (ConA) for α-mannose in N-linked glycans, peanut agglutinin (PNA) for Gaβ1-3GalNAc-R, Maackia amurensis II (MAL-II) for sialic acid with an α2,3 linkage to terminal galactose, Sambucus nigra (SNA) for sialic acid with an α2,6 linkage to GalNAc and Ulex Europaeus Agglutinin I (ULEX-I) for α-linked fucose residues were used.

RNA preparation and gene microarray analysis
Although the action of the inhibitors is known for intermediary metabolism, it is also likely that gene expression is influenced. In order to assess the action of the inhibitors at the genomic level, a gene microarray was employed. In these experiments, the action of inhibitors was examined in PC/AA/C1/SB10C cells. In order to assess the action of the inhibitors at the genomic level, a gene microarray was employed. In these experiments, the presence of the 28S and 18S rRNA on agarose gels and an A260/280 ratio in the range of 1.9–2.1. The A260/280 ratio was determined for all experiments.

A sample (5 μg) of total RNA was used for production of biotinylated cRNA as described in the Affymetrix GeneChip analysis instruction manual (Affymetrix UK, High Wycombe, UK). The human genome 133 A 2.0 array was then hybridized with the biotin-labeled cRNA fragments for 16 h at 45°C. Washing steps for the chip, staining with streptavidin-phycoerythrin, signal-amplification, and scanning were performed according to the manufacturer’s instructions (Affymetrix UK, High Wycombe, UK).

Signal values were exported with the GeneChip operating software (GCSO, Affymetrix).

Further analyses were performed with the software “CorrXpression,” which is described in detail elsewhere (Klein et al. 2005; Wessel et al. 2006). The gene expression data are available at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12422, accession number GSE12422).

Data analysis included the following. Data normalization: the average of each experiment was set in relation to the overall average calculated for all experiments. Differential gene expression: up- and downregulation were defined whenever the expression value of each transcript was higher or lower by at least a factor of 2 in comparison with the controls for each inhibitor experiment.

RT-PCR for core 3 β1-3N-acetylgalactosaminyltransferase
Investigation of the gene profile for the Affymetrix GeneChip 133A 2.0 revealed that the core 3 β1-3N-acetylgalactosaminyltransferase 6 (B3GNT6) gene was not present. Accordingly, RT-PCR was carried out to assess the expression of this gene in PC/AA/C1/SB10C cells and to determine the action of the inhibitors. Total RNA was extracted with RNAlater reagent as described above and reverse transcribed into cDNA with 200 U of superscript RT (Gibco BRL Life Technologies, Paisley, UK) and 25 ng Oligo-dT primers in a final volume of 20 μL enzyme buffer (Gibco BRL Life Technologies, Paisley, UK) for 60 min at 37°C. PCR was performed with 50 ng of cDNA in a 50 μL reaction solution, containing 20 mM Tris, pH 8.0, 50 mM KCl, 2 mM MgCl2, 10 mM of dUTP, 20 pmol of each primer, and 1.25 U of Taq DNA polymerase (Gibco, Paisley, UK). The cycling profile was 94°C for 45 s, 61°C (B3GNT6) or 59°C (GAPDH) for 45 s and 72°C for 45 s for 40 cycles, and a final extension at 72°C for 7 min. The PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The primer sets used for PCR were for B3GNT6: 5'-GGGCCTAGATTCTTTCGAC-3' and 5'-GGGTGCCCCATAGAAAAGA-3' (Iwai et al. 2005) and for GAPDH: 5'-AGTCCACTGGCGTCTTACC-3' and 5'-CGTCAAAGTGGAGAGTG-3'.

Glycosyltransferase assays with mucin substrates and action of inhibitors in colon cell lines and colorectal tissue homogenates
The activity of core 1 synthase, glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase 1, and N-acetylgalactosaminide α-2,6-sialyltransferase was measured in human colonic resection margin mucosal cells, PC/AA/C1/SB10C and HCA7/C29 cell homogenates as before (King et al. 1994). Inhibitors 1, 4, and 7 were included in incubations to give final concentrations of 0.1, 0.5, or 1.0 mM. Controls contained no inhibitor. All incubations were carried out in duplicate in two experiments.

Incubations for the core 1 synthase contained final concentrations of 2 mM UDP-galactose, 4.5 kBq/mL UDP-[14C]galactose, 11.0 GBq/mmol, 0.5 mM ATP, 1 mg/mL BSA, 1.3 mM dimercapto-propan-1-ol, 50 mM MOPS, pH 7.0, 25 mM MnCl2, 0.2% Triton X-100, 0.25 mg total homogenate protein, and 100 μg asialo-OSM (0.25 μmol terminal GalNAc) in a final volume of 100 μL. Incubation was for 30 min at 37°C. Assays were stopped by the addition of 0.5 mL phosphotungstic acid/trichoroacetic acid (5%:15% v/v), mixing and standing for 30 min at 4°C. The suspension was centrifuged at 14,000 g for 5 min at RT and washed twice with 1 mL of 95% ethanol, and finally dissolved in 0.5 mL of 0.2 M KOH before scintillation counting.

Assay for the 2,6-sialyltransferase contained final concentrations of 0.3 mM CMP-N-acetyl-d-neuraminic acid, 27.5 kBq/mL CMP-N-acetyl-d-[14C]neuraminic acid (59.2 MBq/mmol), 100 mM cacodylic acid, pH 6.5, 5 mM EDTA, 0.25% Triton X100, 10 mg/mL BSA, 0.40 mg total membrane protein, and 100 μg asialo-OSM in a final volume of 100 μL. Incubation was at 37°C for 30 min. Assays were stopped with...
0.5 mL PTA/TCA, ethanol precipitation, and scintillation counting as for the core 1 synthase above.

**Data analysis**

Statistical analyses were performed using Dunnett’s t-test and expressed as *P < 0.05, **P < 0.01, ***P < 0.001. NS: not significant.


**Supplementary Data**

Supplementary data for this article is available online at [http://glycob.oxfordjournals.org/].

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**Conflict of interest statement**

None declared.

**Abbreviations**

α-OcHex GalN3, cyclohexyl 2-azido-2-deoxy-α-D-galactopyranoside; α-OcHex GalNAc, cyclohexyl 2-acetamido-2-deoxy-α-D-galactopyranoside; α-OPe GalN3, 2-phenylethyl 2-azido-2-deoxy-α-D-galactopyranoside; α-OPe GalNAc, 2-phenylethyl 2-acetamido-2-deoxy-α-D-galactopyranoside; AOEB, acridine orange and ethidium bromide; BSA, bovine serum albumin; CID, collision-induced dissociation; FBS, fetal bovine serum; GAG, glycosaminoglycan; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption/ionization; nano ESI Q-TOF, nanoelectrospray/ionization quadrupole time-of-flight; O-GlcNAc, O-linked β-N-acetylglucosamine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; sialyl-Lea, sialyl-Leb, sialyl-Lea

**References**


