Action of a library of O-glycosylation inhibitors on the growth of human colorectal cancer cells in culture

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Abstract
O-glycosylation is thought to play a significant role in the regulation of cell growth. However, only limited information is available, and few specific and selective inhibitors have been found. We have synthesized a library of O-glycosylation inhibitors based on benzyl-O-N-acetyl-d-galactosamine. These inhibitors were tested with an established series of human colorectal cancer cell lines, which model the adenoma-carcinoma sequence. Cancer cells were incubated with the inhibitors, and examined for cell growth patterns, and cellular and subcellular glycosylation using a range of lectins with confocal microscopy. The specificity of O-glycan inhibition was confirmed for the library, relative to other forms of glycosylation. All inhibitors tested resulted in smaller cell yields. However, a differential effect on O-glycosylation was detected using the lectins showing variation of localization at a subcellular level in the various cell lines. Further differential action of the inhibitor library was observed for apoptosis and on the cell cycle with the cell lines tested. This work demonstrates that O-glycosylation is closely involved in the regulation of cell growth in colorectal cancer cells and that the generation of a library of low-molecular-mass inhibitors offers a valuable means of examining this regulation at the molecular level.

Introduction
Glycosylation is a major post-translational modification affecting over 50% of all proteins [1,2]. Owing to recent improvements in the technology used to determine glycan sequence information, interest has increased in glycobiology; in particular, in the functional roles of glycans in major biological processes. Glycosylation of proteins occurs in three main groups, the O-glycans, the N-glycans and glycosylphosphatidylinositol anchors [2,3]. The O-glycans themselves can be divided into several groups: first, glycans with GalNAc linked to serine or threonine, often termed mucin-type glycosylation; secondly, GlcNAc linked to serine or threonine (O-GlcNAc) on cytosolic and nuclear proteins; thirdly, fucose and glucose O-linked to serine or threonine in the epidermal growth factor domains in a variety of proteins; fourthly, O-mannosylation found in mammalian proteins such as dystroglycan, and also in the oligomannosyl glycans in yeasts; and finally, the proteoglycans.

The mucin-type glycoproteins represent a major group of glycosylated proteins with important biological functions that remain poorly studied. Part of the reason for this is the absence of specific inhibitors for the biosynthetic pathways leading to the O-glycans. One strategy to investigate the biology of O-glycosylation is to synthesize chemically libraries of compounds known to have biological significance in the metabolic pathways involving these glycans.

Benzyl-O-N-acetyl-D-galactosamine is an inhibitor of the biosynthesis of mucin-type O-glycans, acting as a competitive inhibitor of the monosaccharide–protein glycosidic linkage, GalNAc–α-O-Ser/Thr (Figure 1).

Biosynthesis of O-glycans
The O-glycans are formed by a series of biosynthetic pathways found in the endoplasmic reticulum and Golgi apparatus [1,3]. The primary sequence of the glycans is regulated at the level of substrate specificity of the glycosyltransferases specific for these structures. The addition of the initial monosaccharide GalNAc to the acceptor peptide is catalysed by a family of UDP-N-acetylgalactosamine: polypeptide N-acetylgalactosaminyltransferases (ppGaNTases) [4]. The transfer of GalNAc depends on the arrangement of the serine and threonine residues in the peptide and whether GalNAc has already been transferred to the peptide. Different isoenzymes have characteristic substrate specificities and act in a hierarchical manner to ensure full O-glycosylation of potential sites. Tissue-specific expression of these ppGaNTase isoenzymes generates the typical glycosylation pattern found at the organ/tissue level. Substitution of GalNAc is therefore a pivotal step in the positioning, level of occupancy and glycan extension in defined proteins.

O-glycan structure has been divided into defined regions based on structural data-bank analysis. The linkage of core

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structures to the peptide backbone may be extended through backbone units, and this structure is finally modified by the addition of peripheral units [5]. Generation of the vast array of known O-glycan structures occurs through known biosynthetic pathways involving families of glycosyltransferases with substrate specificities that define the characteristic O-glycan structures [3,5]. Further modification of the glycans also occurs through sulphation of Gal and GalNAc [5], and O-acetylation of sialic acids [6].

Inhibition of O-glycan biosynthesis
Biosynthesis and processing of the mucin-type O-glycans and N-glycans is quite different. As already stated, the synthesis and processing of O- and N-glycans occurs in the endoplasmic reticulum and the Golgi, through the controlled action of a series of glycosyltransferase and glycosidase reactions. N-glycan chains are constructed and transferred as lipid (dolichol) intermediates and processed further by a series of specific glycosidases. In contrast, the O-glycans do not depend on lipid intermediates. The biology of the N-glycans has been elucidated thanks to the use of a family of specific inhibitors that act at different steps in the biosynthetic pathway. No such family of inhibitors exists for the O-glycans.

Inhibition of O-glycosylation has been achieved through the action of a series of glycosyltransferase and glycosidase inhibitors that act at different steps in the biosynthetic pathway. Several compounds with substrate specificities that define the characteristic O-glycan structures [3,5]. Further modification of the glycans also occurs through sulphation of Gal and GalNAc [5], and O-acetylation of sialic acids [6].

Testing the library of O-glycan inhibitors with colorectal cancer cell lines
We have previously examined cancer-related glycosylation in a series of human colorectal cell lines (PC/AA, [11]) derived from a single colonic tubular adenoma with mild dysplasia, and which represents stages of the adenoma–carcinoma sequence identified in human colorectal cancer [12]. This work showed the loss of several glycosyltransferase activities, occurring with the progression to cell lines that were tumorigenic in nude mice [13,14]. Early-passage cells (PC/AA) produced a secreted mucus gel that was largely MUC2, the major intestinal mucin gene, with fewer modifications in glycosyltransferase activities. A pre-malignant cell line (C1) was found to have lost the ability to synthesize a secreted mucus gel. It showed reduced levels of MUC2, and the appearance of MUC5AC, a non-intestinal mucin gene and known marker for adenomas [15], and showed a loss of glycosyltransferases leading to the expression of cancer-related glycan epitopes. A further cell line (10C) was tumorigenic in nude mice and produced no mucus gel, showed low levels of several MUC gene products and had cancer-related glycosylation of secreted mucins [13,14].

These cells provide a valuable model for examining the action of glycosylation inhibitors because their glycosylation pathways have been characterized. The early-passage cells grow very slowly and are not well suited for efficient screening, but both C1 and 10C lines grow rapidly and can be used in this way.

Action of O-glycan inhibitors, a differential action of O- and C-glycosides of benzyl-GalNAc
Examination of a library of uridine analogues mimicking UDP-GalNAc, the central metabolite in O-glycan biosynthesis, revealed several compounds with micromolar Ki values [8] which led to the induction of apoptosis in NIH-3T3 cells [9]. This result demonstrated that mucin-type O-glycosylation may mediate vital developmental programmes.

Using the human colorectal cell lines described above, benzyl-, cyclohexyl- and phenylethyl-O-glycosides of GalNAc and their azido analogues, together with the benzyl- and phenylethyl-α-C-glycosides of GalNAc and their azido analogues, were tested. The action of the inhibitors on the rate of growth, the proportion of shed cells with apoptotic properties, cell-cycle phases, cell-surface glycosylation and
aglycone-glycan formation was measured. Differential action on these parameters was found for the O- and C-glycosides tested, with the benzyl-α-C-glycoside being the most potent in 10C cells.

Conclusion: a role for O-glycosylation in cell growth

The evaluation of O-glycosylation in the regulation of cell growth using two families of inhibitors strongly suggests that cell-growth pathways depend on the correct transfer of O-glycans to proteins responsible for the mediation of these processes. The generation of novel O-glycan inhibitors represents a powerful tool to dissect these pathways.

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References


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