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Modifications on glycosidic enzymes as a strategy to alter tumour cell adhesion

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Als meus Pares,

Resum

Un tret característic del procés carcinogènic es l'alteració de l'expressió i estructura dels carbohidrats i dels enzims que els sintetitzen (glicosidases i glicosiltransferases). Els iminosucres, compostos anàlegs als monosacàrids, s'han descrit com a potents inhibidors de glicosidases pel que han estat proposats com a possible teràpia per inhibir la metàstasis. La incorporació d'una cadena *N*-alquilada als iminosucres sembla incrementar la seva eficàcia, nosaltres hem testat la citotoxicitat de la D-fagomina i del (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol i els seus derivats *N*-alquilat, aquest últims han mostrat una inhibició *in vitro* de la α-L-fucosidases i de la α-D-glucosidases. Els antígens Lewis són oligosacàrids presents en posicions terminals dels carbohidrats de glicoproteïnes i glicolípids, sintetitzats per l'acció seqüencial de fucosiltransferases (FucT) i sialiltransferases (ST). Canvis en l'expressió d'aquests antígens com la sobre-expressió dels antígens sialitzats (sLe^a i sLe^x) faciliten la metàstasis de les cèl·lules tumorals, degut principalment a la interacció entre aquest antígens i la E-selectina present a la superfície de les cèl·lules endotelials. Estudis clínics han demostrat que la inflamació crònica incrementa el risc de carcinogènesis en càncers de l'aparell digestiu. Pel que hem descrit la regulació per citoquines inflamatòries (IL-1β i IL-6) de les fucosiltransferases implicades en la síntesis dels antígens Lewis. Per altra banda hem estudiat la implicació de FucT III i FucT V en la síntesis dels antígens Lewis, i com aquest canvi en el patró d'expressió d'aquests antígens afecta la capacitat d'adhesió de les cèl·lules tumorals.

Abstract

Alterations in the expression of carbohydrates and the enzymes involved in their synthesis (glycosidases and glycosyltransferases) can be considered as universal feature of malignant transformation. Iminosugars, monosaccharide analogues compounds, have emerged as a new class of glycosidase inhibitors and have been suggested as therapeutic tools to inhibit tumour metastasis. The addition of an N-alkyl chain to iminosugars seems to increase their efficiency. We have test the cytotoxicity of D-fagomina and (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol and their N-alkyl derivatives. The N-alkyl derivative have shown an inhibition of α-L-fucosidases i α-D-glucosidases. Lewis antigens are fucosylated oligosaccharides carried by glycoproteins and glycolipids, synthesized by the sequential action of fucosyltransferases (FucT) and sialyltransferases (ST). Their aberrant expression such as the over expression of sialylated antigens (sLe^a and sLe^x) has been viewed as one of the underlying mechanisms for metastasis in different carcinomas, due to the interaction of those antigens with the E-selectin present on endothelial cells that mediate the extravasation of cancer cells. Clinical studies have described that the presence of chronic inflammation in the digestive tract increase the risk of carcinogenesis. Therefore we have described the regulation by pro-inflammatory cytokines (IL-1β and IL-6) of fucosyltransferases involved in Lewis antigen synthesis. In the other hand, we have studied FucT III and FucT V implications in the synthesis of Lewis antigens and how changes on Lewis antigens expression pattern alter the adhesion capacities of MKN45 cells.

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PUBLICATIONS

Mercè Padró, José A. Castillo, Livia Gómez, Jesús Joglar, Pere Clapés, Carme de Bolós. **Cytotoxicity and enzymatic activity inhibition in cell lines treated with novel iminosugar derivatives.** Glycoconj J 2010; 27(2):277-285.

Mercè Padró, Raquel Mejías-Luque, Lara Cobler, Marta Garrido, Marta Pérez-Garay, Sònia Puig, Rosa Peracaula, Carme de Bolós. **Regulation of glycosyltransferases and Lewis antigens expression by IL-1β and IL-6 in human gastric cancer cells.** Glycoconj J. 2011 Mar 2. PMID: 21365246

Mercè Padró, Lara Cobler, Marta Garrido, Laura Camacho, Carme de Bolós. **Down-regulation of FUT3 and FUT5 induces alterations in the expression pattern of Lewis Antigens and reduces adhesive capacities of gastric cancer cells.** (In preparation).

ABBREVIATIONS

Asn, asparagine

CAM, cell adhesion molecules

CGT, ceramide glucosyltransferase

DNJ, Deoxynojirimycin

ECM, Extracellular matrix

EGF, epidermal growth factor

Fuc, fucose

FucT, fucosyltransferases enzyme

FUT, fucosyltransferases gene

Gal, galactose

GalNAc, *N*-acetylgalactosamine

GDP-Fuc, guanosine-diphosphate fucose

Glc, glucose

GlcNAc, *N*-acetylglucosamine

GlcNAcT, *N*-acetylglucosaminyltransferase

H. pylori, *Helicobacter pylori*

HA, Haluronic acid

HUVEC, human umbilical vein endothelial cells

IL, interleukin

IL1R, interleukin-1 receptor

iNOS, inducible nitric oxide synthase

JAK, janus kinase

LacNAc, *N*-acetyllactosamine

Le, Lewis antigens

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MUC, mucin

NF-kB, nuclear factor kB

NeuAc, sialyl acid

POFUT, *O*-fucosyltransferase

Ser, serine

sLe, sialyl-Lewis antigen

ST, sialyltransferase

STAT, signal transducer and activator of transcription

SOCS3, suppressor of cytokine signalling 3

T antigen, Thomsen-Friedenreich

Thr, theonine

VNTR, variable number of tandem repeats

INTRODUCTION

Glycosylation process

Glycosylation is one of the most frequently occurring co- or post-translational modifications made to proteins and lipids in the secretion machinery of the cell (nearly 50% of all proteins are thought to be glycosylated). Glycans are mostly found on the cell surface and extracellular matrix (ECM), and in organellae such as Golgi, endoplasmic reticulum (ER), lysosome, cytosol, and nucleus. As compared to research on DNA, RNA, and proteins, studies on carbohydrates are technically difficult. Thus research in this field has not been underlined for a long period; it also occurs in glycomics as compared to proteomic and genomic research (1-3).

It is well known that glycosylation affects many physicochemical properties of glycoproteins, such as conformation, flexibility, charge, and hydrophobicity. Thus, oligosaccharide modification affects biological processes including receptor activation, signal transduction, endocytosis, and cell adhesion, and leads to the regulation of many physiological and pathological events, including cell growth, migration, differentiation, tumour metastasis, and host–pathogen interactions (4).

It is a highly specific sequential process determined by glycosyltransferases and glycosidases. By manipulating them, it has become possible to modify the oligosaccharide structures and examine the effects of the modification on certain events such as cell adhesion, cell-cell interactions, signalling transduction pathways… (5). Glycosidases are acid hydrolases that catalyze the hydrolysis of the glycosidic linkage to release smaller sugars. They are involved in the biosynthesis and catabolism of glycoconjugates. Glycosyltransferases catalyze trans-glycosylation reactions and are extremely stereospecific, recognize specific sugars, sugar sequences, and often peptide moieties of substrates (6). So far, over 180 glycosyltransferase genes have been identified, by means of genome

sequence data bases and bioinformatics approaches (7). Glycosyltransferases are thought to be arranged in an assembly line in the Golgi, where early acting enzymes are localized in the cis-Golgi, intermediate acting enzymes in the medial-Golgi, and those adding terminal structures in the trans-Golgi.

Glycosylation produces different types of glycoconjugates that are typically attached to proteins or lipids (Figure1). The carbohydrate structure of glycoproteins can be *N*-glycans, *O*-glycans, and glycosaminoglycans (frequently termed proteoglycans). In *N*-glycans the first sugar is attached to the amide group of an asparagine (Asn) in a consensus sequence Asn-X-Ser/Thr, where -X- may be any aminoacid except proline. In *O*-glycans the carbohydrate structure is bound to a hydroxyl group of a serine (Ser) or a theonine (Thr) residue (8). Although in glycosaminoglycans the carbohydrate structure is also linked to serine or threonine, they are linear, and often highly sulphated (such as heparan sulphate and chondroitin sulphate) (9). In glycosphingolipids (often called glycolipids) the sugar structure is usually attached via glucose or galactose to the terminal primary hydroxyl group of the lipid moiety ceramide, which is composed of a long chain base (sphingosine) and a fatty acid (Maccioni et al., 2002).

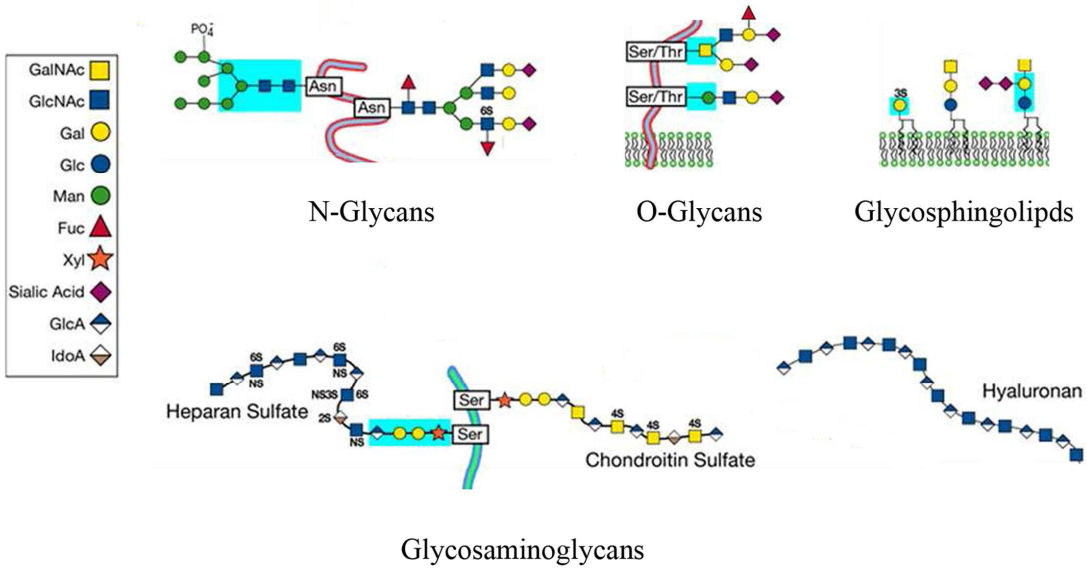


Figure 1. Different type of glycoconjugates, glycoproteins divided in *N*-glycans, *O*-glycans, and proteoglycans as heparin sulphate or free glycosaminoglycans such as hyaluronan; and glycolipids known as glycosphingolipids. Extracted and modified from the *Essentials of Glycobiology* book.

N-Glycans

N-glycosylation is initiated in the lumen of the ER by the transfer of a preassembled oligosaccharide (Glc3Man9GlcNAc2) from a lipid precursor (dolicyl pyrophosphate) to Asn residues in nascent polypeptide chains (10). The precursor glycan Glc3Man9GlcNAc2-Asn is sequentially trimmed by glucosidases and mannosidases before acting as potential glycosyltransferases that lead to complex and hybrid-type chains. This process is performed in ER and Golgi apparatus where a continuous quality control of glycosylation process takes place.

Processing or trimming of Glc3Man9GlcNAc2-Asn begins with the sequential removal of glucose residues by α-glucosidases I and II (11). Both

glucosidases act in the lumen of the ER. Afterwards the glycoprotein is transferred to the cis-Golgi. In the cis- and medial-Golgi the activity of α-mannosidase II, III and *N*-acetylglucosaminyltransferase I and II (GlcNAcT-I, II) takes place. As a result of this activity the precursor for all biantennary complex N-glycans is synthesized (12). In the trans-Golgi the extensive array of mature complex *N*-glycans are obtained. This part of the biosynthetic process can be divided into three components: (1) sugar additions to the core, (2) elongation of branching *N*-acetylglucosamine residues by sugar additions, and (3) "capping" or "decoration" of elongated branches.

1. The main core modification is the addition of fucose in an α1–6 linkage to the N-acetylglucosamine adjacent to asparagine in the core. This occurs by the action of FucT VIII (13;14).

2. In the elongation process two types of glycans are formed; type I, which are mostly restricted to epithelia of gastrointestinal and reproductive tracts and type II, which constitute the majority of N-glycan. They are produced by the addition of a β-linked galactose residue to the initiating *N*-acetylglucosamine structure. The β-galactose linkage can be Galβ1-3 for type I structure or Galβ1-4 for type II carbohydrates.

3. The most important "capping" or "decorating" reactions involve the addition of sialic acid, fucose, galactose, *N*-acetylgalactosamine, and sulphate to the branches. Capping sugars are most commonly α-linked and therefore protrude away from the β-linked *N*-acetylglucosamine structure. Many of these structures are shared by *N*- and *O*-glycans and glycosphingolipids. In this steps Lewis antigens are synthesized by the action of fucosyltransferases and sialyltransferases.

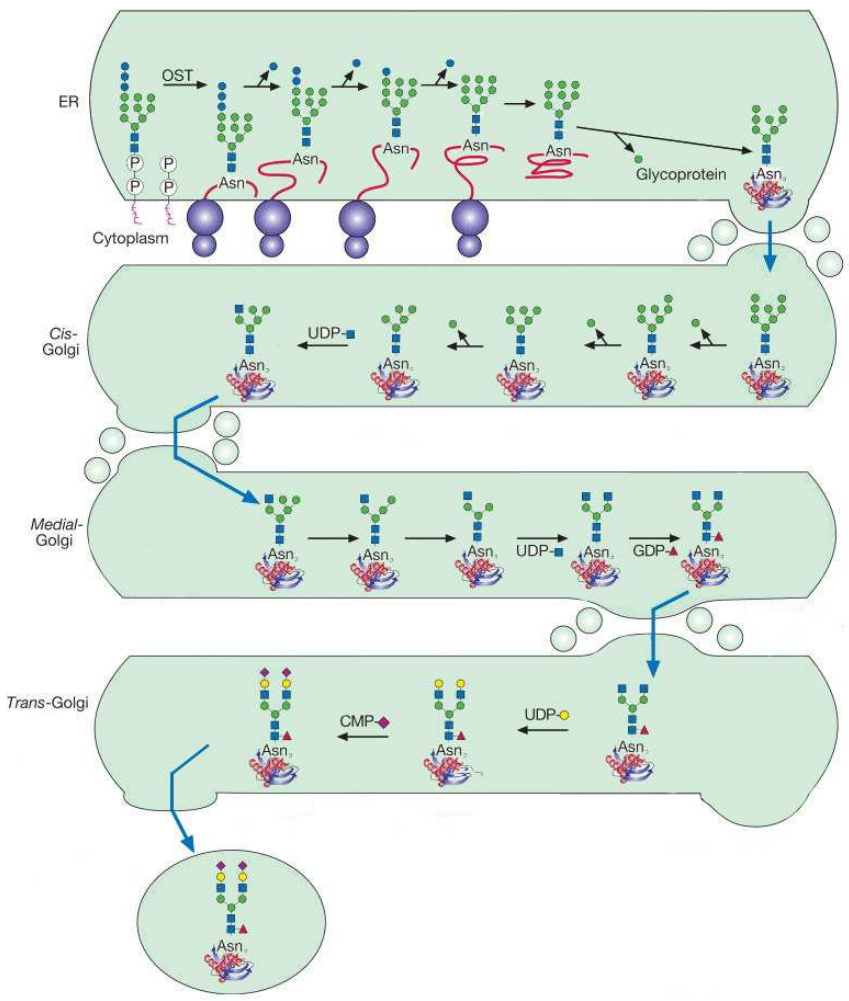


Figure 2. Processing and maturation of *N*-glycans. Dolicyl-pyrophosphate is transferred to Asn-X-Ser/Thr sequences in the ER where glucosidases remove the three glucose residues, and mannosidase removes a mannose residue. The sugar additions, the branching *N*-acetylglucosamine and the capping take place in the Golgi apparatus. Extracted and modified from the *Essentials of Glycobiology* book.

O-Glycans

Glycoproteins with *O*-glycosylation (mainly mucins) are found in secretions and on the cell surfaces. In the *O*-glycan pathways, every sugar is transferred from a specific nucleotide sugar donor by the action of specific membrane-bound glycosyltransferases. *O*-glycosylation is initiated in the cis-Golgi by addition of *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to the hydroxyl groups in serine and threonine residues. This reaction is catalyzed by a large family of up to 20 different polypeptide GalNActransferases (15;16), and results in the formation of the GalNAcα1-Serine/Threonine or Tn antigen. Once is synthesized, the further glycosylation of Tn antigen can be performed along different pathways. These reactions depend on the activity of specific glycosyltransferase enzymes and the availability of precursor substances. The addition of sialic acid by an α2-6 sialyltransferase forms the STn antigen (SAα2,6GalNAcα-O-Ser/Thr) and this structure does not apparently undergo further glycosylation. The addition of galactose by a β1-3 galactosyltransferase results in the formation of the Thomsen-Friedenreich antigen (Galβ1-3GalNAcα-O-Ser/Thr) also known as T antigen. This disaccharide is also called Core 1 structure. Most *O*-glycan structures found in glycoproteins are based on the Core 1 structure. Individuals with the Tn syndrome are deficient in this β1,3 galactosyltransferase resulting in the expression of Tn antigen on their hematopoietic cells (17).

Core 1 structures can be further elongated by the action of different glycosyltransferases that add different monosaccharides such as galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) or sialic acid (NeuAc). The action of sialyltransferases on T and Tn antigens generate sialylated antigens known as tumour associated antigens (ST, STn…). From T antigen can be generated the core 2 antigens, and from Tn antigen can be synthesized core 3 to 8 structures. Two main intermediary *O*-glycan

structures are found in mucins: Type I (GlcNAcβ1-3 Galβ1-3)n and Type II chains (GlcNAcβ1-3 Galβ1-4)n. When fucose or sialic acid are added by fucosyltransferases or sialyltransferases, respectively, the carbohydrate chain cannot be further elongated.

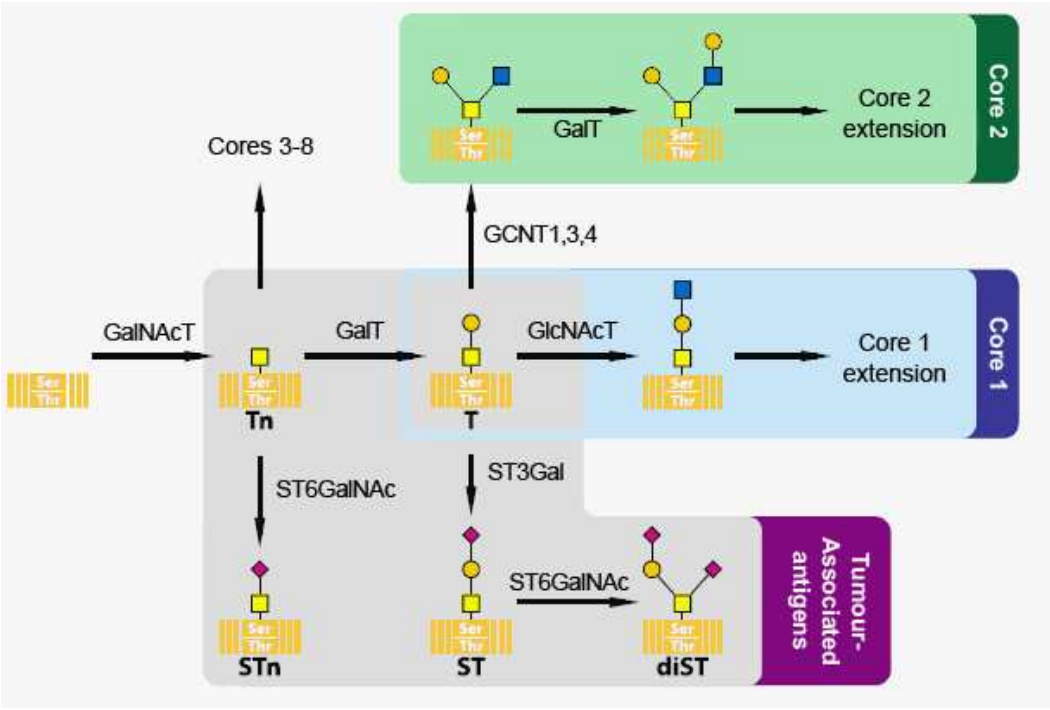


Figure 3. *O*-glycosylation is initiated with Tn antigen synthesis by GalNActransferases action. T antigen is afterwards obtained, from these antigens all the range of *O*-glycans is synthesized. Extracted and modified from (18).

Glycosyltransferases

Fucosyltransferases

Fucosyltransferases (FucT) catalyze the reaction in which a fucose residue is transferred from the donor guanosine-diphosphate fucose (GDP-

Fuc) to the acceptor molecules including oligosaccharides, glycoproteins, and glycolipids (19). Fucosylated glycoconjugates are involved in a variety of biological and pathological processes. The regulation of fucosylation appears to be complex, and depends on the type of cells or organs involved.

Fucosylation is one of the most important type of glycosylation in cancer. In 1979 Hakomori presented the first paper in which fucosylation and cancer were related. In this study, the authors compared the fucosylation levels of glycolipids in hepatoma cells and normal hepatocytes (20) .

The FUT gene family encodes a group of proteins (FucTs) that show a complex tissue- and a cell type–specific expression pattern. This expression pattern varies during development and malignant transformation. FucTs display different but sometimes overlapping enzymatic properties. They are the responsible of Lewis antigens synthesis, which are fucosylated oligosaccharides carried by glycoproteins and glycolipids in the terminal position of the carbohydrate chains. Some of them have been shown to be essential for normal biological function. At present 13 fucosyltransferase genes have been cloned (FUT1-FUT13) (21;22).

Classification of fucosyltransferases

Based on the site and link of fucose addition, FucTs are classified into α1-2 fucosyltransferases (FUT1-2), α1-3/4 (FUT3-7,9), α1-6 (FUT8) and *O*-fucosyltransferases (FUT12-13) named in human POFUT1 and POFUT2 respectively (Table 1). For FUT10 and 11 their fucosyltransferase activity has not been confirmed (23). Fucosyltransferases, except *O*-FucTs, are type II transmembrane Golgi-anchored proteins containing an *N*-terminal cytoplasmic tail, a transmembrane domain, and an extended stem region followed by a large globular C-terminal catalytic domain facing the Golgi lumen (24). *O*-FucTs are soluble proteins with ER localization (25;26).

Fucosyltransferases	Activity	Lewis antigens products
FucT I	α1-2	H-type 2
FucT II	α1-2	H-type 1
FucT III	α1-3, α1-4	Lewis b, Lewis a, sialyl-Lewis a Lewis y, Lewis x, sialyl-Lewis x
FucT IV	α1-3	Lewis x, (Lewis y, sialyl-Lewis x)
FucT V	α1-3, α1-4	Lewis b, Lewis a, sialyl-Lewis a Lewis x, sialyl-Lewis x
FucT VI	α1-3, (α1-4)	Lewis x, sialyl-Lewis x
FucT VII	α1-3	Sialyl-Lewis x
FucT IX	α1-3	Lewis x
FucT VIII	α1-6	-
O-FucT I	FucαSer, FucαThr	-
O-FucT II	Unknown	-

Table 1. Fucosyltransferase family classification.

- α1-2 Fucosylation

There are two polymorphic genes (FUT1 and FUT2). Both genes form a cluster (within 35 kb) on human chromosome 19 (19q13.3) and protein products (FucT I and FucT II) share an homology of 67% in amino acid sequence(27). They transfer GDP-fucose in an α1-2 linkage to terminal Gal residues in *N*- or *O*-glycans. They have shown a specific pattern distribution in normal tissues. For instance, in stomach, cells in the superficial epithelium express FUT2, while deep gland cells express FUT1 (28).

- α1-3/4 Fucosylation

Six different genes (FUT3-7, 9) are described with distinct enzymatic properties and tissue expression patterns (29;30). Chromosomal localization studies have demonstrated that the FUT3, 5, and 6 genes form a cluster (within 35 kb) on human chromosome 19 (19p13.3) (31). In addition, their protein products (FucTs III, V, and VI) have a high degree of sequence similarity (~90% identity), which suggests that the human FUT3-FUT5-FUT6 cluster was generated by duplication events. The sequences of FUT3, FUT5, and FUT6 are highly polymorphic. Several inactivating mutations have been described explaining the frequent occurrence of negative phenotypes for these enzymes and their different tissue distribution. FucT III is found mainly in the gastrointestinal tract and kidney (32). FucT V is minimally expressed in normal tissues (29;33). FucT VI also known as plasma-type (34;35) is mainly found in plasma, but also in kidney and liver (36). Although they share high homology, they possess different acceptor substrate affinities and specificities.

FUT4 encodes the myeloid-type enzyme FucT IV (37-40) although it is widely expressed in many different tissues (41). FUT4 gene is found at 11q21 (42), but its implication in biological processes remains unclear. FUT7, encoding leukocyte-type FucT VII, it is expressed at high levels in hematopoietic cells. FUT7 is located in human chromosome 9 (9q34.3) (43;44), FucT VII controls leukocyte trafficking through an essential role in L-, E- and P-selectin ligand biosynthesis. In 1996 Maly found that FUT7 knockout mice had abnormality extravasation of leukocyte during inflammation (45). FUT9, which encodes brain-type FucT IX (36) is located in 6q16 (46). FUT9 knockout mice showed the disappearance of the Lewis x structure in the brain and increased anxiety-like behaviour (47;48).

- α1-6 Fucosylation

FUT8 is the only gene with α1-6 fucosylation activity, and encodes FucT VIII. It is located in chromosome 14q23/24 and it is widely expressed in mammalian tissues (49;50). It catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of *N*-glycans, being the responable of core fucosylation in mammals (50;51).

- *O*-Fucosylation

Two *O*-fucosyltransferases have been described. They are able to transfer fucose directly to Ser/Thr residues of polypeptides and are very important for signal transduction via Notch receptor (52). Nonetheless, POFUT1 and POFUT2 share several biochemical properties that distinguish them from other known fucosyltransferases (53;54). For instance, both enzymes catalyze the addition of fucose directly to a serine or threonine in proteins instead of to another sugar. The EGF (epidermal growth factor) repeats, in Notch receptor, are modified by two types of *O*-glycosylation: *O*-glucosylation and *O*-fucosylation; both are important for Notch activity. Notch signalling is deregulated in many cancers (55).

Sialyltransferases

Sialyltransferases (STs) are type II transmembrane glycoproteins that predominantly reside in the trans-Golgi compartment (56). The different sialyl-linkages are elaborated by different members of the sialyltransferase family. These glycosyltransferases share the same donor substrate (CMP-sialic acid) but may differ in the glycosidic structure on which they act and in the type of glycosidic linkage they form. Despite the relatively small number of existing sialyl-linkages, 20 mammalian sialyltransferases have been cloned. As described for fucosyltransferases, a certain degree of redundancy exists for sialyltransferases, the same glycosidic linkage can often be elaborated by different gene products (57).

They can be further classified into three families: α2-6 sialyltransferases mediate the transfer of sialic acid with an alpha 2,6-linkage to terminal galactose (ST6Gal I, II) or to *N*-acetylgalactosamine (ST6GalNAc I-VI), α2-8 sialyltransferases mediate the transfer of sialic acid with an alpha 2,8-linkage to another sialic acid (ST8Sia I-V), and α2-3 sialyltransferases are the responsible of Lewis antigens synthesis transferring sialic acid to galactose residue with an alpha 2,3-linkage (ST3Gal I-VI) (Table 2). ST3Gal IV and VI transfer sialic acid to residues located mainly on type II structures (Galβ1-4GlcNAc), and ST3Gal III catalyzes the transfer of sialic acid with an alpha 2,3-linkage to terminal Gal residues located on either Galβ1-3GlcNAc or Galβ1-4GlcNAc structures (58).

Sialyltransferases	Acceptors
ST3Gal I	Galβ1-3GalNAc
ST3Gal II	Galβ1-3GalNAc
ST3Gal III	Galβ1-3(4)GalNAc
ST3Gal IV	Galβ1-3GalNAc, Galβ1-4GalNAc
ST3Gal V	Galβ1-4Glc-Cer
ST3Gal VI	Galβ1-4GlcNAc
ST6Gal I	Galβ1-4GlcNAc
ST6Gal I	Galβ1-3(4)GlcNAc
ST6GalNAc I	GalNAc, Galβ1-3GalNAc, Siaα2-3 Galβ1-3GalNAc
ST6GalNAc II	GalNAc, Galβ1-3GalNAc, Siaα2-3 Galβ1-3GalNAc
ST6GalNAc III	Siaα2-3 Galβ1-3GalNAc
ST6GalNAc IV	Siaα2-3 Galβ1-3GalNAc
ST6GalNAc IV	GM1b
ST6GalNAc VI	GM1b, GT1b
ST8Sia I	GM3
ST8Sia II	Siaα2-3 Galβ1-3GlcNAc
ST8Sia III	Siaα2-3 Galβ1-4
ST8Sia IV	Sia Galβ1-4GlcNAc
ST8Sia V	GM1b, GD1a, GT1b, GD3

Table 2. α2-3 sialyltransferases, α2-6 sialyltransferases and α2-8 sialyltransferases families and their principal acceptors.

Several experimental and clinical studies have related the presence of sialic acids and cancer. Morgenthaler (59) reported a correlation between the high levels of sialylation on the surface of murine cancer cell lines and their invasive properties. In clinical studies it has been shown an increase in sialyltransferase levels in serum of cancer patients (60). The expression of sialylated antigens in gastrointestinal cancer cell is reported to correlate with tumour progression and distant metastasis to the liver (61).

Lewis antigens

Lewis antigens are fucosylated oligosaccharides carried by glycoproteins and glycolipids in the terminal position of the carbohydrate chains. These antigens are biochemically related to humans ABH blood groups which are formed by the sequential addition of fucose, sialic acid, galactose and *N*-acetylgalactosamine to the backbone carbohydrate chains (62). They are not only found on human erythrocytes, but also expressed in many other cell types such as epithelia, where mucins are the major carriers of these antigens. Much of the recent interest in these antigens has resulted from the observation that they undergo specific changes during tissue embryonic development and, less predictably, during malignant transformation.

The presence of Lewis antigens in the cell surface has been related to many processes of intercellular recognition and adhesion or cell-matrix interactions such as implantation, embryogenesis, tissue differentiation, tumour metastasis, inflammation and bacterial adhesion (63-65). Therefore structural alterations in this terminal glycan epitopes are always accompanied by changes in biological properties of cells, such as the interaction between E-selectin and sLe^a and sLe^x antigens expressed on the surface of leukocytes this interaction is considered an important step for the successful recruitment of leukocytes into tissue (58;66;67). These fucosylated glycoconjugates synthesized can be used by bacteria as a source of nutrients (68) and can serve as receptors for their adhesins (69). In pathological process Lewis antigens are important in bacterial adhesion in the first stages of cell infection. Other illustration of Lewis antigens repercussion is the implication in the bacterial adhesion in the first stages of cell infection.of Lewis b and sialyl-Lewis x in the bacterial adhesion at the first stages of cell infection. In gastric mucosa, it has been reported that the adhesion of *H. pylori* to the superficial epithelium cells is mediated by

Lewis b and sialyl-Lewis x expressed in epithelial cells (70). Another case is the capacity of *Bacteroides thetaiotaomicron* to ensure the production of host fucosylated glycoconjugates that could affect the ability to other components of the normal flora to establish a stable niche or could affect the vulnerability of the intestine to colonization by pathogens (71).

Lewis antigens synthesis

Lewis antigens are synthesized from two types of backbone structures, containing galactose (Gal) and *N*-acetylglucosamine (GlcNAc). Type I terminal antigens are Lewis b (Le^b), Lewis a (Le^a), and sialyl-Lewis a (sLe^a) and are synthesized from Galβ1-3GlcNAc precursor and, type II terminal antigens are Lewis y (Le^y), Lewis x (Le^x), and sialyl-Lewis x (sLe^x) and are synthesized from the Galβ1-4GlcNAc precursor. The synthesis of Lewis antigens takes place by the sequential action of several fucosyltransferases and sialyltransferases on the two precursor structures (Figure 4 and 5).

The first step of Lewis antigens synthesis is catalyzed by α1-2 fucosyltransferases and α2-3 sialyltransferases. FucT II and ST3Gal III synthesize preferably type I antigens although can also synthesize type II structures but with low efficiency. FucT I, ST3Gal IV and ST3Gal VI synthesize mainly type II antigens (72). The α1-3/4 fucosyltransferases (FucT III-VII and IX) catalyze the final step of Lewis antigens synthesis. It remain unclear which α1-3/4fucosyltransferase is the responsible of each Lewis antigen synthesis, since their activities are altered in malignant transformation and vary between tissues. However from enzymatic studies using synthetic carbohydrate acceptors the specific activities of α1-3/4 fucosyltransferases have been identified. α1-3 activity (type II antigens synthesis) was detected in FucT III-VII and IX, while α1-4 activity (type I antigens synthesis) was only detected in FucT III and FucT V. FucT III has

a predominant α1-4 activity, whereas FucT V adds fucose with the same efficiency to type I and type II structures (73) (Dupuy F 2004). Thus, only FucT III and Fuc V catalyze the last step of type I antigens synthesis. In type II Lewis antigens synthesis can be performed by FucT III-VII and IX, most of them are tissue specific and show different affinities in Lewis antigens synthesis. However, from enzymatic studies and published data an estimated scheme can be generated (Figure 4 and 5).

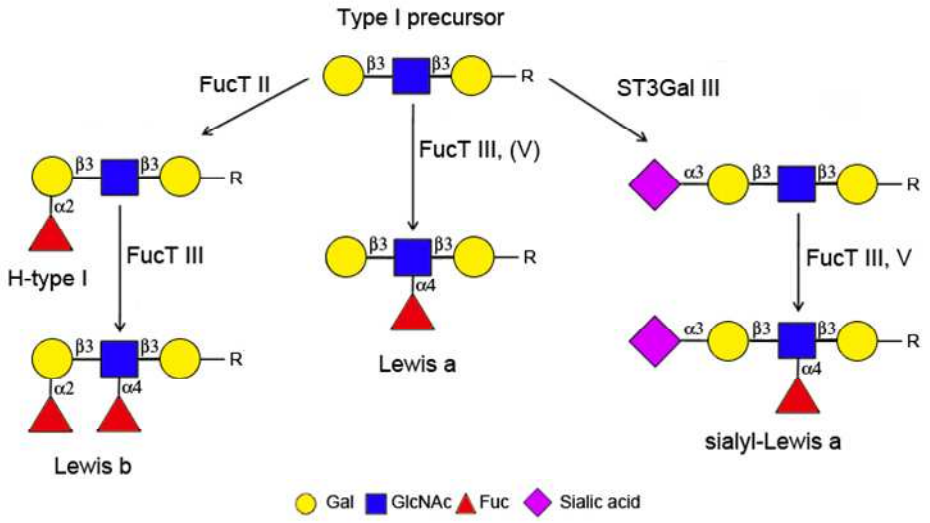


Figure 4. Type I Lewis antigens synthesis

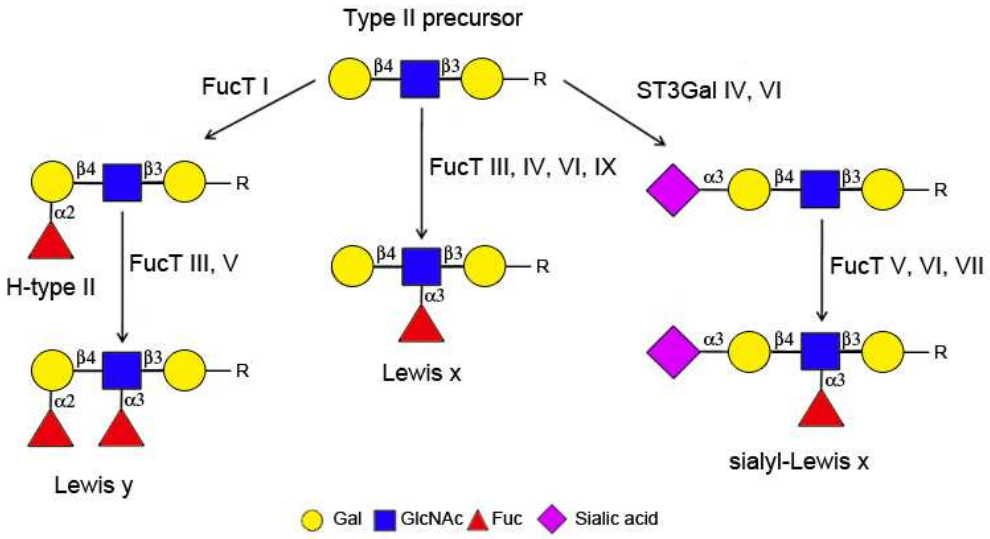


Figure 5. Type II Lewis antigens synthesis

Glycosylation associated with carcinogenesis

Alterations in the expression and structure of carbohydrates can be considered as an universal feature of malignant transformation. Altered glycosylation is present mainly on the surface of the cell, affecting important cellular and molecular processes such as cell adhesion, cell growth and proliferation, cell-cell interactions, division, differentiation and signal transduction mechanisms.

Modifications on carbohydrate metabolism, alterations on enzymes involved in the synthesis and degradation of carbohydrates (glycosyltransferases and glycosidases) have been associated with the carcinogenic process. Several studies have been described enzymatic alterations in malignant tissues compared to normal tissues. Cancer cells use carbohydrate moieties to escape recognition by immune cells when migrate

through the body. In this sense fucosylation and sialylation processes affecting Lewis antigens synthesis have been reported to be altered during the malignant transformation. Therefore, it can be used as serum tumour markers for cancer diagnosis, such as sLe^a known as CA19-9 or sialyl SSEA-1 or sLe^x known as CD15. sLe^a is used as a marker in cancers of digestive organs whereas sLe^x is used as a marker in cancers of lung, breast and ovary (74). All type II Lewis antigens (Le^x, Le^y and sLe^x) have a high prevalence in breast cancer, and can be also considered as a tumour markers (75).

Based on the enormous potential of sugar structures to code biological information and the presence of lectins, proteins that specifically recognizes and binds the glycans structure, it is evident that protein–carbohydrate interactions play a crucial role in recognition events. In particular, carbohydrate detecting lectins are described to play a role in the metastatic process, in which they may act as homing receptors for tumour cells. The aberrant expression of Lewis antigens has been viewed as one of the underlying mechanisms for metastasis in different carcinomas, due to the role of sialylated antigens (sLe^a and sLe^x) in the interaction between leukocytes and cytokine-activated endothelial cells through the adhesion molecule E-selectin. It has been suggested that sLe^a and specially sLe^x can mediate carcinoma cell – endothelial cell interactions in a similar manner as leukocyte – endothelial cell interaction (76). E-selectin is not always expressed on vessel wall, but some cancer cells have the ability to induce its expression on endothelial cells. The blood vessels near cancer nests frequently express E-selectin (77), probably due to the presence of inflammatory cytokines such as IL-1β or TNF-α that have the capacity to induce the expression of E-selectin on human endothelial cells (Figure 6).

Hematogenous metastasis of cancer is a complicated process consisting of multiple steps. The process starts with the intravasation of

cancer cells into the blood stream in the primary tumour lesion. Cancer cells then travel in the blood stream and finally adhere to endothelial cells somewhere in the peripheral vessel walls enter into connective tissue and form a new metastatic lesion. The adhesion process starts with the interaction between sialyl Lewis antigens expressed on the surface of cancer cells and selectins expressed on endothelial cells. The second step is the process of implantation of cancer cells into the monolayer, mediated mainly by β1-integrins. The efficiency of the initial adhesion step seems to greatly affect the overall efficiency of cancer cell invasion to endothelial cells (78) (79).

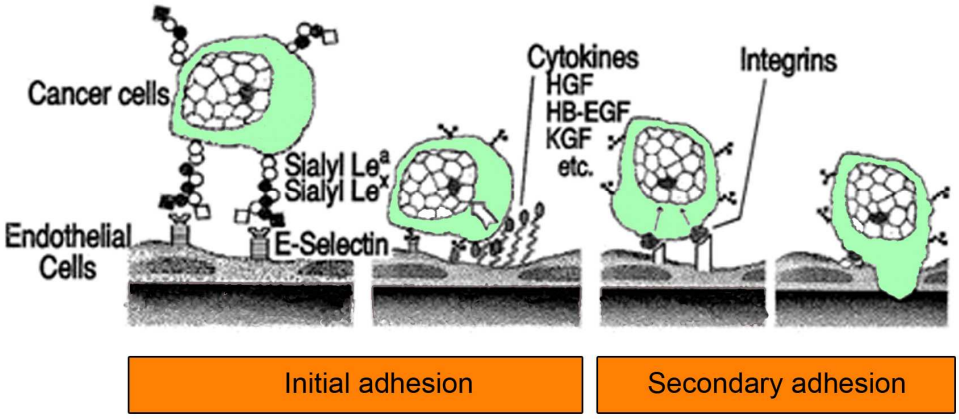


Figure 6. Schematic representation of the extravasation process of cancer cells at vessel walls. The initial adhesion is mediated by E-selectin and Lewis antigens binding, afterwards cytokines stimulate cancer cells to activate integrins which mediated the secondary adhesion. Extracted and modified from (78)

OBJECTIVES

OBJECTIVES

Glycoconjugates containing fucose, such as Lewis antigens, play important roles in cell adhesion and cell-cell interactions, being fundamental in the metastatic process. Thus, the aim of the study was to modify glycosidic enzymes to induce changes on Lewis antigens expression and consequently alter adhesive capacities of cancer cells.

Our **first objective** was to evaluate the effects of *N*-alkyled iminosugars derivatives on glycosidases activity and their cytotoxicity in cancer cell lines.

Our **second objective** was to study the regulation of fucosyltransferases and sialyltransferases by pro-inflammatory cytokines and its effect on expression levels of Lewis antigens in gastric cancer cells.

Our **third objective** was to analayze the down-regulation of FUT3 and FUT5 by shRNA strategy on gastric cancer cells, and their effects on Lewis antigens expression pattern and in the adhesion capacities of tumour cells.

CHAPTER 1

Inhibition of glycosidases by novel iminosugars derivatives

INTRODUCTION

Glycosidases

Glycosidases are a class of hydrolytic enzymes that catalyze the hydrolysis of the glycosidic linkage from oligosaccharides to release smaller sugars. They are extremely common enzymes with many different roles in nature, they are mainly involved in the degradation of oligosaccharides (e.g. sucrose, lactose, starch) as well as in the biosynthesis and catabolism of glycoconjugates as glycoproteins, glycolipids, glycosaminoglycans. These enzymes are crucial in many biological processes such as eukaryotic glycoprotein processing, polysaccharide and glycoconjugate anabolism and catabolism. Deficiencies in the catabolic activity of glycosidases can alter *N*-glycans structure affecting the maturation and transport of glycoproteins and glycolipids. Therefore lysosomal glycosphingolipidoses disorders characterized by a specific enzymatic deficiency lead to lysosomal storage of glycosphingolipids. Because of their importance, many efforts have been devoted to control glycosidase activity by means of selective inhibitors (80).

Carbohydrates containing fucose are often involved in a number of important physiological processes. Several pathogenic events, including inflammation, cystic fibrosis, and cancer, have been associated with an abnormal distribution of α-fucosidases. The human L-fucosidase has been shown to be a diagnostic serum marker for the early detection of colorectal and hepatocellular cancers (81;82).

Hydrolysis mechanism of glycosidic enzymes

Glycosidases can be classified according to the stereochemical outcome of the hydrolysis reaction in retaining or inverting glycosidases, depending on the final configuration of the anomeric centre in reference to stereochemistry of the original glycosidic linkage. The hydrolysis reaction

in the retaining glycosidases operates through a double-displacement mechanism (e.g. α-amylase) and consist of two steps, each one passes through an oxocarbenium ion-like transition state (Figure 7). The hydrolysis reaction in the inverting glycosidases employ a single displacement mechanism (e.g. β-amylase) and consist of one step involving also oxocarbenium ion-like transition states (Figure 8). The reaction occurs with acid/base and a nucleophilic assistance, in the case of retaining glycosidases, provided by two amino acid typically glutamate or aspartate (83). The difference in the hydrolysis mechanism results from the distance between the residue acting as a nucleophile, in the retaining mechanism, and the bound substrate.

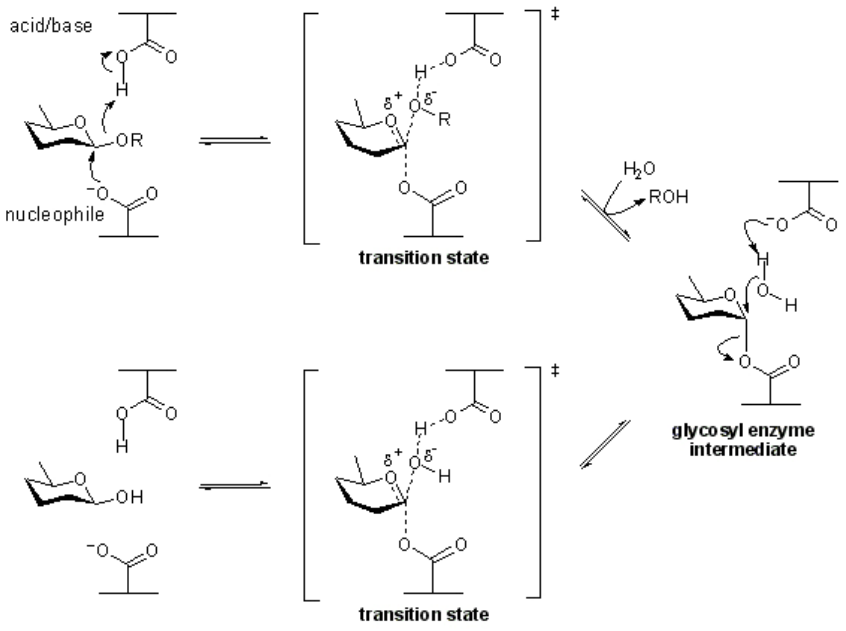


Figure 7. Hydrolysis mechanism with net retention of anomeric configuration of β-glycosidases. Adapted from Withers S and Williams S. "Glycoside hydrolases" in CAZypedia.

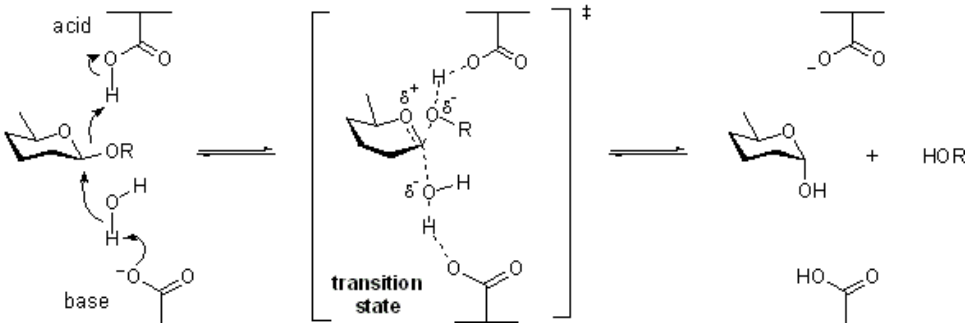


Figure 8. Hydrolysis mechanism with net inversion of anomeric configuration of β-glycosidases. Adapted from Withers S and S Williams S. "Glycoside hydrolases" in CAZypedia.

Glycosylation and deglycosylation processes, performed by glycosyltransferases and glycosidases, occurs through dissociative transition states with significant oxocarbenium ion character. The iminosugars, natural monosaccharide analogues, have been postulated as a potent glycosidases inhibitor due to mimic the charge distributions of oxocarbenium ion transition state structures.

The implication of glycosidases in tumourigenesis

Analysis of putative alterations in carcinogenesis of glycosidases has been carried out by several studies. These studies have shown that a large number of glycosidases are increased in serum, primary cancerous and metastatic tissues of cancer patients. It has been described an increase of *N*-acetyl-β-glucosaminidase in serum of gastric cancer patients (84) and an increase of β-D-Galactosidase and α-L-Fucosidase in serum of

patients with colon adenocarcinomas (85). These results suggest that increased glycosidase activities may be involved in cell transformation to primary cancerous, (possibly through degradation of fundamental membrane-associated components) and metastatic propagation. In this context, glycosidase activities have been found to be increased in cancer tissues from lung, brain, ovarian, colon, prostate and stomach cancer.

Iminosugars molecules

Iminosugars are monosaccharide analogues in which the endocyclic oxigen has been replaced by a nitrogen. Several iminosugars isolated from natural sources are selective inhibitors of specific glycosidases and therefore they have a significant therapeutic interest in the treatment of several diseases (86). In the last decade, several synthetic derivatives of iminosugars have emerged as a major new class of very potent glycosidase inhibitors. It is generally accepted that these inhibitors interact with the catalytic mechanism of glycosidases by mimicking the carbocationic form of glycosidase transition state. The first molecule of this family, named isofagomine, was reported by Bols (87) and proved to be a very potent β-glucosidase inhibitor. Meanwhile, several other sugar analogues with nitrogen at the anomeric position have been prepared and used to treat several diseases (88). As an example, derivatives from deoxynojirimycin compounds have been clinically tested to treat the type I of Gaucher disease (89;90), as well as effects in the inhibition of α-glucosidase activity of several iminosugars are being used as therapeutic agents in type 2 diabetes (91).

In tumour cells, iminosugars, that act as competitive inhibitors of specific glycosidases, have been suggested as therapeutic tools to inhibit tumour metastasis (reviewed in (92)). As an example, gem-diamine 1-N-iminosugars have been reported to suppress invasion of B16 melanoma

and 3LL lung carcinoma cells (93), and swainsonine has been reported to shut down the carbohydrate processing pathway prior to the initiation of β1-6GlcNAc linked branch by inhibiting the Golgi R-mannosidase II. Swainsonine treatments lead to inhibition of tumour cell metastasis, decrease of solid tumour growth in mice, and enhancement of cellular immune response. Nonetheless, the inhibition of a related catabolic R-mannosidase in lysosomes makes its clinical use less desirable (94).

D-fagomine, ((2*R*,3*R*,4*R*)-2-hydroxymethylpiperidine-3,4-diol), a polyhydroxylated piperidine analogue is a naturally occurring iminosugar that has been reported to have inhibitory activity against α-, β-glucosidase and α-, β-galactosidase from mammals (95). The conformation of this iminosugar mimics the stereochemical conformation of D-glucose and D-mannose monosaccharides at C3, C4 and C5 (Figure 9). Additionally, the (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (96) is an iminosugar that competes with the natural substrate (a fucose glycoside or fucose-GDP) by mimic its charge distribution and hydroxyl group topography at the transition state of the biocatalytic reaction. It has been described as a specific inhibitor of α-L-fucosidase from bovine kidney (97) (Figure 10).

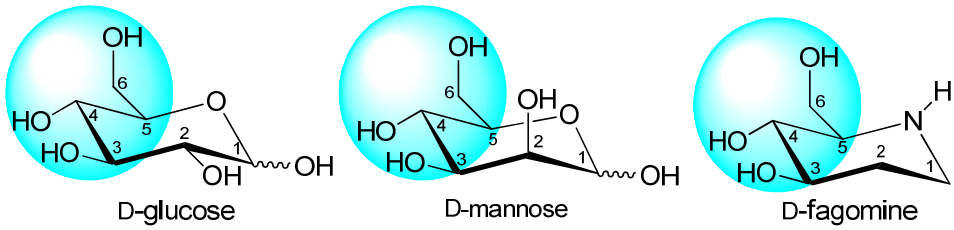


Figure 9. Structure of the iminosugar D-fagomine and the monosacharides D-glucose and D-mannose

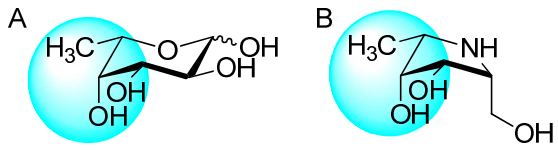


Figure 10. (A) Structure of fucose monosaccharide and (B) the iminosugar (2R,3S,4R,5S)-2-(hydroxyl-methyl)-5-methylpyrrolidine-3,4-diol.

N-alkylation chain addition on iminosugars

N-alkylation of iminosugars has been postulated as a requirement in the inhibition of several glycosidases, as it was reported by Platt. Studies, where DNJ (deoxynojirimycin) compound needs a *N*-alkyl chain of at least three carbon atoms to inhibit CGT (ceramide glucosyltransferase) (98). In in vitro assays with isolated CGT an increase in inhibition up to 10-folds were described when the N-alkyl chain was increased from C4 to C8 (99). The hydrophobic properties of *N*-alkyl chain help the cell to uptake iminosugars. In *in vivo* experiments, the uptake of the iminosugars is augmented proportionally to the increase of chain length (99). Even though compounds with very long chain (C16 and C18) are kept in the membrane and do not reach the cytoplasm (100). The presence of *N*-alkyl iminosugars with long chains at high concentrations can generate plasma membrane disruption due to a detergent-like effect (101).

This *N*-alkylated iminosugars have been used in treatments of lysosomal glycosylation disorders that have been previously presented as examples of iminosugar applications (102;103). *N*-butyl-DNJ inhibits the ceramide-specific glucosyltransferase and α- and β-glucosidases (104). This capacity to inhibit ceramide-specific glucosyltransferase has been used in order to reduce the synthesis of glycolipid substrate in Gaucher disease patients and provides a demonstrable clinical improvement (105).

Also, the inhibition of the *N*-acetyl β-hexosaminidase, important in osteoarthritis, has been reported to be enhanced in compounds with longer *N*-alkyl chain (106).

RESULTS

Padró M, Castillo JA, Gómez L, Joglar J, Clapés P, de Bolós C. Cytotoxicity and enzymatic activity inhibition in cell lines treated with novel iminosugar derivatives. Glycoconj J. 2010; 27(2): 277-85.

CHAPTER 2

Regulation of glycosyltransferases and

Lewis antigens expression by IL-1β and IL-6

INTRODUCTION

Mechanisms of inflammation in Gastric carcinogenesis

Inflammation is an important environmental factor that promotes tumourigenesis and the progression of established cancerous lesions. The functional relationship between inflammation and cancer is not new. In 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation. Chronic inflammation may increase the risk of neoplastic transformation through several mechanisms: increased oxidative stress causing DNA damage and alterations in cell cycle regulation, changes in expression of oncogenes and tumour suppressor genes, inhibition of apoptosis with maintained proliferation and interruption of cell adhesion mechanisms.

Tumours arise in the context of stroma, which includes lymphocytes, myeloid cells, fibroblasts and connective tissue. This cells have a remarkable ability to produce a variety of factors and small chemicals that can influence tumourigenesis, by promoting the growth and survival of tumours, angiogenesis, tissue invasion and metastases (107). Clinical studies have shown a particularly association between chronic inflammation and cancer in the digestive tract where the risk for carcinogenesis increases in presence of chronic inflammatory conditions such as esophagitis, gastritis, colitis, pancreatitis, and hepatitis (108). It has been demonstrated that pluripotent cell recruitment to gastric mucosa from the bone marrow plays a key role in repair but also in carcinogenesis (109). The mechanisms underlying mucosal gastric cancer, generating from bone marrow derived cell are not yet understood.

Inflammation induced by *H. pylori* infection

H.pylori is a Gram-negative, spiral shaped, microaerophilic bacilli that colonizes the gastric epithelium and represents the most common bacterial infection worldwide (110). It is well accepted that *H. pylori* infection is a major factor in the pathogenesis of gastric cancer. Since 1994, when the International Agency for Research on Cancer classified *H. pylori* as a Group 1 human carcinogen, a large number of studies have been published confirming this association. It causes chronic gastric mucosal inflammation, characterized by the presence of infiltrating macrophages, B and T lymphocytes, polymorphonuclear cells and plasma cells (111). Gastritis is the basic process that mediates *H. pylori*-induced damage, and its extension and distribution determines the clinical outcome. In patients with chronic gastritis, atrophic changes of the gastric glands gradually spread over time as a result of inflammation (112). This change is important for gastric cancer development. It has been reported that a protein isolated from the bacterial body of *H. pylori* stimulates macrophages in the epithelium and promotes the secretion of inflammatory cytokines such as TNF-α or IL-1β (113).

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is the main cause of inflammation in this type of infections. *H. pylori* has been recently reported to increase growth of gastric tumours via LPS-TLR4 signalling (114), that is able to activate MAPK and NF-κB pathways (115). Approximately 20% of *H. pylori* in the stomach is found adhered to the surfaces of mucus epithelial cells. The adhesion of the bacteria to the epithelial layer is assured by a large family of 32 related outer membrane proteins, including adhesins. These proteins bind to specific macromolecule receptors on the epithelium. This adherence may be advantageous to *H. pylori* by helping to stabilize it against mucosal shedding into the gastric lumen and ensuring good access

to nourishing exudate from gastric epithelium that has been damaged by the infection (116). One of the best characterized adhesins is BabA (70). That protein binds to gastric epithelial cells through Le^b antigen forming a scaffold apparatus. Strains possessing the BabA2 gene adhere more tightly to epithelial cells, promoting an aggressive phenotype. These strains are associated with higher incidence of gastric adenocarcinoma (117). Another important outer membrane protein is SabA, which interacts with sLe^x antigen. This interaction although it is weaker than the one performed by BabA, plays an important role in the *H. pylori* adherence (Figure 11) (118).

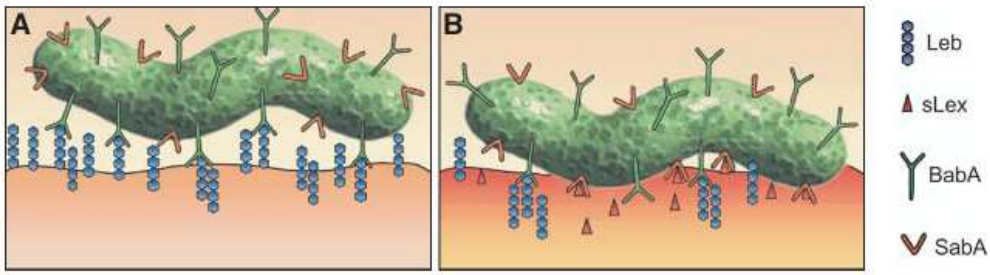


Figure 11. Adhesion of *H. pylori* to gastric epithelium. This figure illustrates the proficiency of *H. pylori* for adaptive multistep mediated attachment. (A) *H. pylori* adherence through BabA to Le^b antigen expressed in glycoproteins in the gastric surface of epithelium. *H. pylori* uses BabA for strong and specific recognition of the Le^b antigen. (B) During persistent infection and chronic inflammation, *H. pylori* induces changes in glycosylation patterns of epithelial cells to up-regulate sLe^x antigens. Then, SabA binds to sLe^x antigens leading to close attachment of *H.pylori*. Extracted and modified from (118)

In gastric mucosa, the expression of sialylated glycoconjugates (sLe^x and sLe^a) is increased in gastritis. Studies on Rhesus monkeys confirmed that gastric epithelial sialylations are induced in *H. pylori* infections under inflammatory conditions (118). In accordance with this,

high levels of sialylated glycoconjugates have been found in *H. pylori*–infected persons, which decreased after eradication of infection and resolution of gastritis (119).

Pro-inflammatory cytokines associated with gastric cancer development

Pro-inflammatory cytokines are able to regulate genes and glycoproteins involved in the gastric neoplastic transformation, as it occurs with intestinal mucins. MUC2 and MUC4 are not detected in normal gastric epithelia but are present in gastric adenocarcinomas. Recently it has been reported that MUC2 can be regulated by TNF-α and IL-1β through the NF-κB signalling pathway (120) and MUC4 is activated by IL-6 through the gp130/STAT3 pathway (121). The molecular pathways associated with TNF-α, IL-1β and IL-6 is detailed next.

IL-1β and TNF-α signalling pathway

TNF-α is a strong tumour promoter (122), mainly produced by activated macrophages and lymphocytes during inflammation. TNF-α is also produced by tumours and can act as an endogenous tumour promoter (123). Increased levels of TNF-α in *H. pylori* patients have been detected (124;125). TNF-α stimulus has been linked to all steps involved in tumourigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Its regulatory effects are mainly mediated through the NF-κB pathway.

IL-1β is a potent pro-inflammatory cytokine inhibitor of gastric acid secretion that contributes to the initiation and amplification of the inflammatory response to *H. pylori* infection (126). To analyse the

etiological role of IL-1β in gastric carcinogenesis a transgenic mouse model over-expressing IL-1β in the stomach has been recently established (127). IL-1β transgenic mice developed spontaneous inflammation, metaplasia, dysplasia and carcinoma of the stomach, demonstrating that increased levels of IL-1β can be enough to induce gastric neoplasia. Furthermore, IL- 1β may also play an important role in metastasis. In this sense IL-1β has been reported to be able to enhance invasiveness of gastric cancer cells through NF-κB activation (128).

In unstimulated cells, NF-kB is sequestered in the cytoplasm and complexed with IKB proteins. When cells are stimulated, TNF-α or IL-1β bind to their receptors (TNFR1 or IL1R) and form TRAF/RIP complexes. This interaction triggers IKK activation, leading to phosphorylation, ubiquitination and degradation of IκB proteins, allowing NF-kB dimmer (p65/p50) to translocate to the nucleus and bind to κB sites in the promoters of target genes related with tumour progression, metastasis, angiogenesis and antiapoptosis (Figure 12). Most of these genes overlap with the target genes of STAT1 and STAT3, activated by IL-6 stimuli. Since the promoter region of many of these genes contains GAS (STAT-binding elements) in addition to kB sites (NF-kB binding element). These target genes are often additively or synergistically activated. Several studies revealed that NF-kB is constitutively activated in numerous types of carcinoma, including pancreatic, breast, colorectal, hepatocellular and gastric carcinomas. In stomach, NF-κB activation is an important event for the progression from chronic inflammation to carcinogenesis. In this sense, the degree of gastritis has been described to be modulated by NF-κB activation induced by H. pylori infection (129;130).

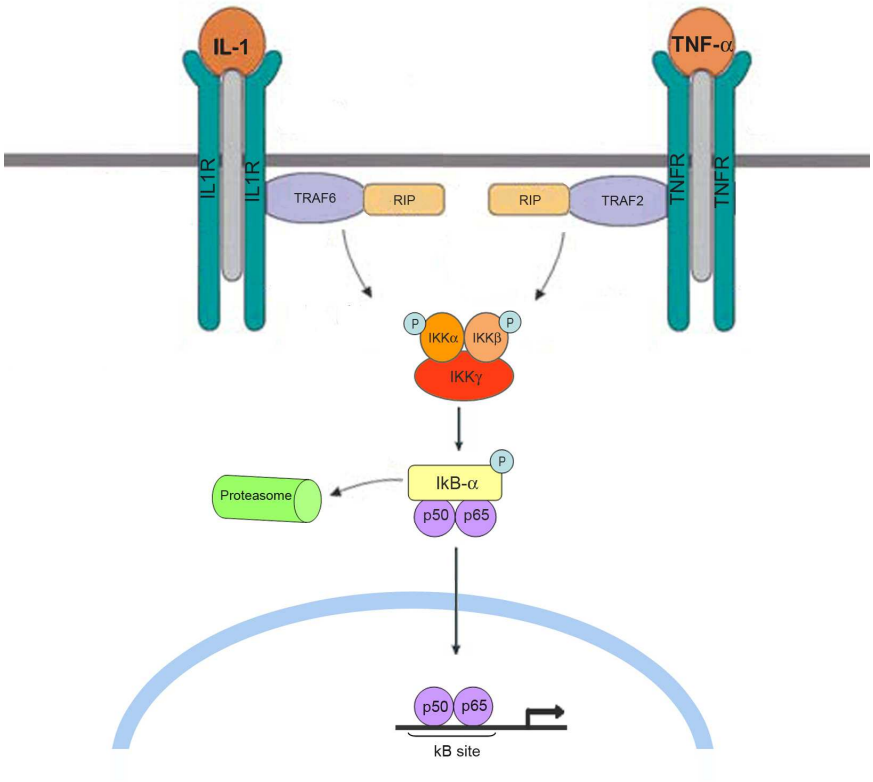


Figure 12. IL-1β and TNF-α signalling pathway. When NF-kB(p50/p65) and IKB protein are complexed, NF-kB is retained in the cytoplasm. The signalling pathway activation through TRAF6/RIP and IKK proteins release IKB from NF-kB allowing p50/p65 to translocate to the nucleus.

IL-6 signalling pathway

IL-6 is a pleiotropic cytokine that is important for immune responses, cell survival, apoptosis and proliferation. In gastric carcinogenesis, IL-6 has been associated with the disease status and outcomes of gastric tumours (131;132). IL-6 initiates signalling by binding to its receptor (IL-6Rα), then gp130 is recruited activating autophosphorylation of Janus kinases. JAKs also phosphorylate several specific tyrosine residues on the intracellular domain of gp130. These phosphorylation sites of gp130 act as docking sites for SHP-2 and STATs

transcription factors, activating two different signalling pathways: SHP-2/ras/MAPK/ ERK1/AP-1 signalling cascades and JAK/STAT pathway. These two pathways under normal conditions are in homeostatic balance (Figure 13). JAK/STAT pathway is initiated by phosphorylations of STATs transcription factors (STAT1 and STAT3) leading to homo- or heterodimers formation. These dimmers translocate to the nucleus and activate target genes (133). This signalling is mainly mediated by STAT3. A significant increase in STAT3 activation has been detected in *H. pylori* infected patients and in gastric adenocarcinoma (134), and it has been proposed as a prognostic factor for poor survival of gastric cancer patients (135). STAT3 regulates the expression of genes involved in suppressing apoptosis (Bcl-2), promoting angiogenesis (Vegf, Mmp9) and inducing proliferation (c-myc). MAPK cascades starts by the recruitment and phosphorylation of SHP-2 which links the Grb2/SOS complex to gp130. This binding allows the activation of Ras, which starts Ras/Raf/MAPK cascade ending in gene regulation by transcription factors activation as AP-1. The negative regulator of STAT3 activation, suppressor of cytokine signalling 3 (SOCS3), can bind to phospho-tyrosine residues of gp130 and block the union of other transcription factors. SOCS3 gene is induced via JAK/STAT, acting as a classical feedback pathway inhibitor. It modulates the balance between the two pathways (136).

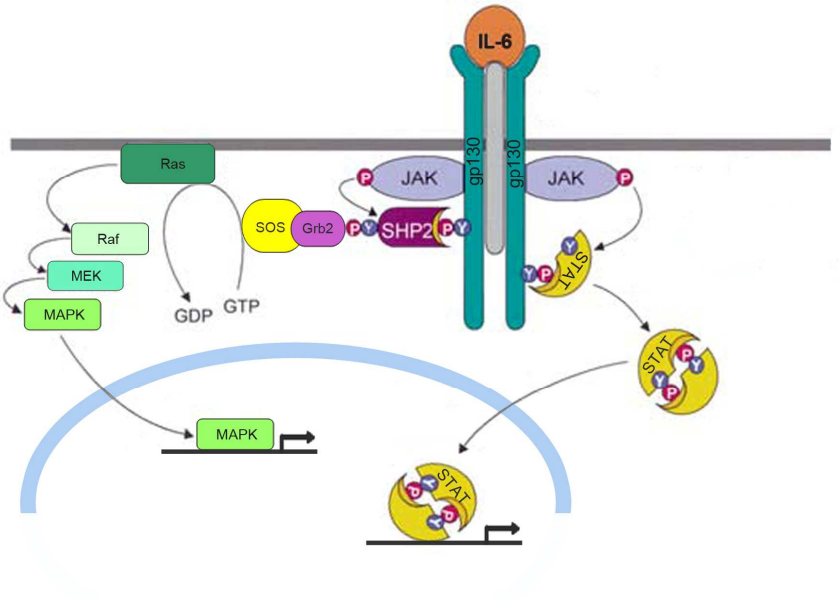


Figure 13. Representation the two major pathways activated by IL6 signalling, the MAPK signalling cascades and JAK/STAT pathway. Adapted from (137).

To study the consequences of disrupting the balance between the STAT1/3 and SHP2/MAPK pathways Ernst *et al.* generated a mouse model of gastric cancer. The mice (gp130 (757F/ F) mice) carry a point mutation (Y757F) that disrupts SOCS3 and SHP2-binding on the IL-6 family receptor gp130 (138). As a result, these mice show hyperactivation of STAT3, resulting in chronic gastric inflammation and distal stomach tumours that resembles human intestinal-type adenocarcinoma (139). These studies suggest an important role of the STAT3 system in gastric carcinogenesis. Using this genetic background several mutant mice for other members of the IL-6 cytokine family have been obtained with the purpose to elucidate the contribution of IL-6 members to gastric cancer development and progression (140;141).

RESULTS

Padró M, Mejías-Luque R, Cobler L, Garrido M, Pérez-Garay M, Puig S, et al. Regulation of glycosyltransferases and Lewis antigens expression by IL-1β and IL-6 in human gastric cancer. Glycoconj J. 2011; 28(2): 99-110.

CHAPTER 3

Role of FUT3 and FUT5 in Lewis antigens expression and in the adhesive capacities of gastric cancer cells

INTRODUCTION

Implication of fucosylation in cell adhesion

Aberrant glycosylation of glycoproteins expressed in tumour cells has been underlined as an essential mechanism in cell adhesion. Glycoconjugates containing fucose have been shown to play important roles in cell adhesion and cell-cell interactions. As previously mentioned, carcinoma cells frequently present an enhanced expression of sialyl-Lewis antigens (sLe^a and sLe^x) which could be essential factors in the adhesion to the endothelium and in the formation of metastasis (76). There are clear evidences that fucosyltransferase activity is altered in tumour tissues as reported in (142). In pancreatic tumour cell lines have been reported a decrease of α1-2 fucosyltransferase activity and an increase of α1-4 fucosyltransferase activity (142). Previous studies have show that alterations in fucosyltransferases expression in tumour cell lines modify the Lewis antigen expression pattern and the adhesive capacities of tumour cells (143).

It is important to understand the biosynthesis of sialyl-Lewis antigens and any mechanism that regulates their synthesis in order to modify the metastatic phenotype of tumour cells. The impairment of sialyl-Lewis antigen expression could become a good strategy in the development of anti-adhesion treatments to prevent metastasis.

However, the direct correlation between the expression of fucosyltransferases and the expression of sialyl-Lewis antigens in gastrointestinal tissues has not been elucidated yet. Moreover, the biosynthesis of sialyl-Lewis antigens can be strongly distinct in different tissues and can be deeply altered by neoplastic transformation (144).

Suppression of specific fucosyltransferases by the RNAi technique will not only help to elucidate the function of fucosylated

antigens in carcinogenesis, but it would also provide a new approach for the treatment of cancers through inhibiting Lewis antigen synthesis. In this context Weston reported in 1999 that the metastatic phenotype of a well-characterized adenocarcinoma cell line can be altered by stable expression of antisense sequences of the human α1-3/4 fucosyltransferase gene, and he suggested FUT3 as a principal fucosyltransferase involved in sLe^a and sLe^x synthesis in colon cancer cells (145). Further studies confirmed Weston's reports: In pancreatic cancer cells, transfections with a FUT3 antisense sequence induced decrease in sLe^a expression associated with the loss of the metastatic phenotype (146). In colon carcinomas, inhibition on proliferation has been reported by using FUT3 and FUT6 antisense sequences (147). *In vivo* models down-regulating FUT1/4 expression described a decrease in Le^y antigen expression and an inhibition of cancer growth (148). In gastric cancer cells the knowledge about fucosyltransferases and their implication in Lewis antigens synthesis is very limited, although there are some studies with overexpression of fucosyltransferases, no reports silencing or down-regulating fucosyltransferases have been published.

Glycoproteins implicated in adhesion

One of the most important features of tumour cell invasion is the ability to establish or modulate adhesion to other cells or to an extracellular matrix, a process mediated by a large number of adhesion proteins. Cell adhesion molecules (CAMs) are cell surface glycoproteins that have a large extracellular domain, a transmembrane domain, and a cytoplasmic functional domain. These molecules are named adhesion molecules because of their relatively strong binding to specific ligands. Although cell adhesion proteins are involved in cell–cell or cell–matrix interactions, they have also been shown to be involved in cell motility,

cell migration, differentiation, cell signalling, and gene transcription. Due to these important functions they are proposed to be involved in pathological conditions including tumour progression and metastasis. As we have already mentioned this invasive phenotype is often related with changes on the oligosaccharides structures. Therefore, it is not surprising that aberrant glycosylation patterns can serve as markers in cancer disease.

E-cadherin

E-cadherin is a transmembrane glycoprotein that belongs to the classical cadherin family of Calcium-dependent adhesion. It is the main epithelial cell-cell adhesion molecule and represents a key member of the adherens junction. It is well-known that epithelia-to-mesenchymal transition, a process associated with normal development, wound healing, cancer progression and metastasis, is associated with loss of E-cadherin expression (149). Studies on E-cadherin expression in gastric tumours show that decrease of E-cadherin expression correlate with tumour invasion (150). E-cadherin can be post-translationally modified by phosphorylation, *O*-glycosylation and *N*-glycosylation. *N*-glycosylation can regulate the cell-cell adhesion mediated by E-cadherin (151). Cytoplasmic *O*-glycosylation has been shown to occur in response to ER stress and inactivates E-cadherin by preventing its transport to the cell membrane (152).

Mucins (MUC1)

Mucins are extracellular glycoproteins of high molecular weight that maintain epithelial integrity and lubricate and protect epithelial surfaces. Mucins can be classified into two main categories, membrane-associated and secreted. Secreted mucins are entirely extracellular whereas membrane-associated mucins are bound to the cells by an integral

transmembrane domain. The cytoplasmic tails can be linked to cytoskeletal elements and can participate in signal transduction (153). The structural feature shared by all mucins is the tandem-repeat domain, containing tandem repeats of identical or highly similar sequences rich in serine, threonine and proline residues. Variation in the specific sequence and number of tandem repeats is observed among different mucins and among orthologus mucins from different species. The repetitive domain is characterized by an interindividual variable number of tandem repeats (VNTR). The VNTR polymorphism is caused by the instability of the number of repetitions from generation to generation (Figure14) (154).

Mucin glycosylation is predominantly O-linked and occurs on serines and threonines inside the tandem repeats (155). These carbohydrate structures might be one of the defining characteristics for mucins functionality. MUC1 has 5 potential glycosylation sites within each tandem repeat (156). MUC1 has been identified to be one of the principal glycoproteins carrying sialyl-Lewis antigens on tumour cells (157).

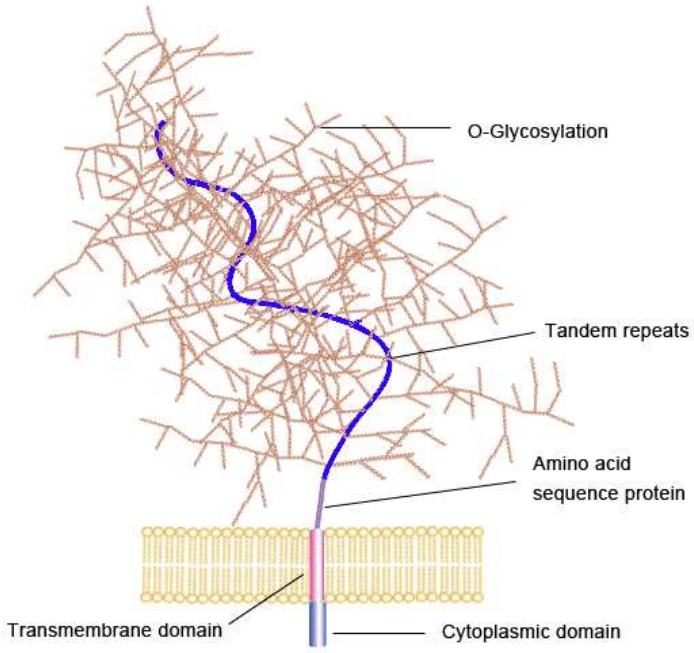


Figure 14. Mucin structure schematic representation of MUC1.

Mucins are known to play important roles in carcinogenesis in several ways. Cis-interactions modulate adhesion or mask potential adhesion-mediating receptors (155); trans-interactions on the other hand, conveyed either by mucins displayed on the cell surface or by the secreted form, act as ligands for some of the same receptors on cells they get in contact with. This duality in mucins function is central to their proposed role in metastasis. Distant metastasis requires dissociation of normal adhesion at the primary site and establishment of new adhesive interactions at the colonized tissue. Mucins are able to participate in both processes (18). An altered expression of mucins has been observed in tumours from epithelial origin and in pre-cancerous lesions. For instance, MUC1 expression is a hallmark of most breast carcinomas, MUC4 of

pancreatic cancer, MUC16 of ovarian cancer and MUC2 of intestinal metaplasia (158).

CD44

The CD44 glycoproteins are well characterised members of the hyaluronate receptor family of cell adhesion molecules. The principal ligand of CD44 is hyaluronic acid. Hyaluronic acid is a negatively charged, high-molecular-weight glycosaminoglycan present mostly in the extracellular matrix of connective tissues and it contains repeating disaccharide units of D-glucuronic acid (β1-3) and N-acetyl D-glucosamine (β1-4).

- CD44 protein structure

This glycoprotein is produced from a single gene by both alternative splicing and post-translational modification. All the isoforms belonging to this family are encoded by one single gene present on chromosome 11 in humans. All isoforms contain a constant region comprising a large ectodomain (270 amino acids), a transmembrane domain (23 amino acids) and a cytoplasmic domain (72 amino acids) (Figure 15). These regions are encoded by the first 5 exons (s1-s5) and the last five exons 16–20 (s6-s10). Conjoined expression of these 10 exons leads to the smallest and most abundant isoform, known as CD44s or CD44H. This isoform is expressed by a large number of non-epithelial cells. Close to the transmembrane region, a variable part encoded by various combinations of exons 6–15 (v1–v10) gives rise to CD44 variant isoforms known as CD44v or CD44E, the epithelial form (159;160).

The N-terminus (s1-s5) contains at least five conserved N-glycosylation sites, and two chondroitin sulphates. In the membrane proximal extracellular region several potential O-linked glycosylation sites can be found, and in the standard extracellular region there are

several potential sites for carbohydrate modification such as heparan sulphate, keratin sulphate, and sialic acid residues. The alternatively spliced forms also have extensive potential modification sites, including serine/threonine rich regions for O-glycosylation (161).

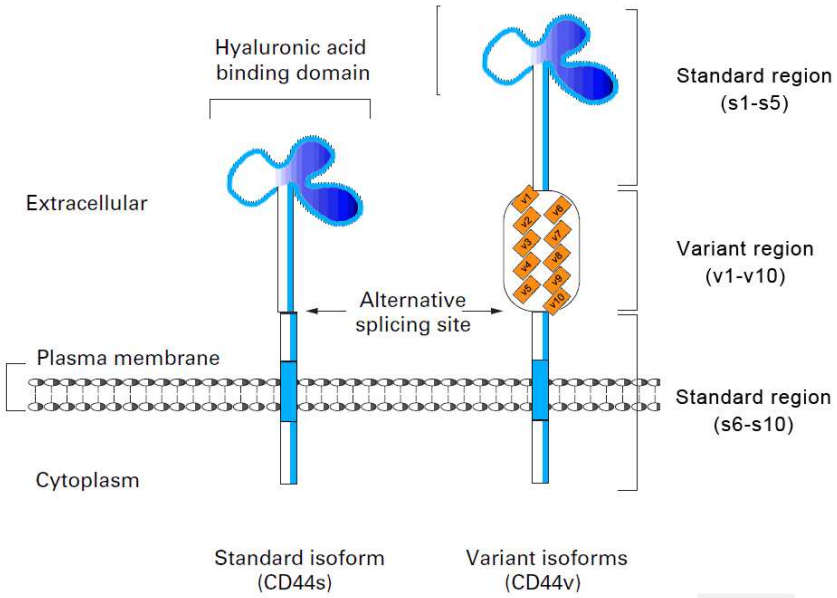


Figure 15. CD44 protein structure. The standard isoform binds its principal ligand, hyaluronic acid at the N-terminal, distal extracellular domain. Sequences from up to 10 alternatively spliced variant exons (vl-v10) can be inserted in the proximal extracellular domain. The cytoplasmic domain interacts with the cytoskeleton. Adapted from (162).

- Glycosylation on CD44

CD44 is expressed in a very diverse assortment of cell types. Each cell lineage, at each stage of differentiation or activation, must regulate the function of the receptor to suit its own requirements. Although all CD44 isoforms contain the hyaluronic acid recognition site, not all cells expressing CD44 bind the hyaluronic acid ligand constitutively. Cell specific carbohydrate modifications of the extracellular domain have

emerged as an important mechanism for regulating the HA binding function of CD44 probably by altering the folding or charge of the receptor molecule (163;164). Therefore it seems likely that glycosylation may promote different CD44-associated adhesive properties in different cell types (165).

The binding of CD44 to hyaluronic acid is regulated by glycosylation in at least four different ways as four distinct oligosaccharide structures were described by Skelton in order to effect the hyaluronic acid binding. The terminal α2,3-linked sialic acid on N-linked oligosaccharides inhibite the binding; the first N-linked N-acetylglucosamine residue increase the adhesion; N-acetylgalactosamine incorporation into non–N-linked glycans augmented hyaluronic acid binding; and O-linked carbohydrate chains on N-deglycosylated CD44 enhanced binding. The negative charges on the O-linked glycans have been described as potentially perturbing and/or directly interfering with the two clusters of positively charged residues which form the HA-binding site located at the N-terminus of all CD44 isoforms (163).

- CD44 linked with cancer

During inflammatory response and in relation with tumour growth an overproduction of hyaluronic acid has been found in the surrounding of tumour cells (166). Therefore, the differential expression of CD44 is likely to be an important determinant of tumour cell biology (167).

Initial studies showed that tumour tissues contained a number of unusual CD44 transcripts relative to those present in corresponding normal tissues (168). In addition, tumour cell lines with raised concentrations of CD44 proteins were shown to be capable of forming more aggressive tumours in animal experiments (169;170). CD44 has been described as a determinant in the progression to the metastasis stage

in vivo using pancreatic cancer cells in rats (171). At the same time, studies employing antisense CD44 and treatment of cells with anti-CD44 antibody blocked the invasive capacities of human lung carcinoma cells (172).

The process of metastasis is complex and likely to require multiple tumour cell properties. A number of human tumours appear to utilize variant isforms of CD44 (CD44v) during carcinogenesis and tumour progression (173). In gastric cancer, the expression of CD44v6 and CD44v5 is up-regulated and in addition, expression of CD44v5 is preferentially found in poorly differentiated carcinomas and metastatic lymph nodes (174).

The capacity to bind hyaluronic acid does not differ much between CD44s and CD44v. However, it has been recently reported that CD44v, but not CD44s, carries the sialyl-Lewis x antigen, which serves as a good ligand for selectin in human colon cancer LS174T cells (175;176). A supporting fact in this context would be that the variant domain of CD44 is particularly rich in O-glycosylation sites. Subsequently, studies indicated that the sialyl-Lewis antigens are also carried preferentially by CD44v compared to CD44s in some other cancer cells (177). The CD44v molecule carrying sialylated Lewis antigens plays a dual function in cell adhesion (Figure 16): serving as a ligand for selectins through its terminal glycans structures attached to the variant domain, while contributing to cell adhesion through its classical binding to hyaluronic acid.

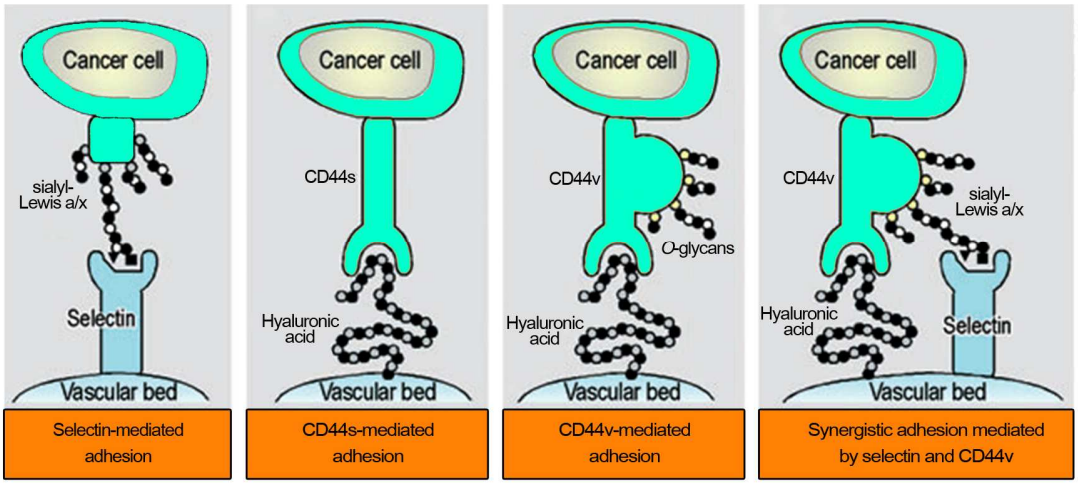


Figure 16. Schematic illustration of the dual function of CD44v carrying sialyl-Lewis antigens. Both CD44 isoforms can bind equally hyaluronic acid and are involved in the migration process of tumoural cells. CD44v carries additional sialyl-Lewis a and sialyl-Lewis x antigens, and their interaction with E-selectin facilitates the extravasation of tumour cells. (Adapted from (178))

MATERIALS AND METHODS

Cell lines culture

Cell lines Human gastric cancer cell line MKN45 was obtained from ATCC and GP220 was established at Dr. Sobrinho-Simoes laboratory [F. Gartner, (1996)]. HEK293T was obtained from our institute cell bank and HUVEC (human umbilical vein endothelial cells) was kindly provided by Dr Cerutti. These cell lines were cultured in DMEM supplemented with 10% FBS and maintained at 37ºC in 5%CO2 atmosphere. Cells were routinely checked for Mycoplasma contamination (VenorGeM, Minerva Biolabs, Germany).

Infection of shFUT3 and shFUT5

Short hairpin RNAs (shRNAs) against human FUT3 and FUT5 and scramble were ordered from MISSION® shRNA plasmids (Sigma). Five different sequence of shRNA for each gene were tested. Lentiviral particles were produced in 293T HEK cells and after infection of MKN45 and GP220 cells, stable cell lines expressing the shRNA were selected with puromycin at 3,5µg/ml in MKN45 cells and at 0,5µg/ml in GP220 cells. The efficiency of mRNA down-regulation and the specificity for each sequence was assessed by qRT-PCR.

RNA extraction and RT-PCR

Total RNA extraction was carried out from cultured cells using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St Louis, MO). After rDNAse I (Ambion) treatment, mRNA levels of FUT3 and FUT5, were quantified by triplicate using QuantiTect SYBR green RT-PCR (Qiagen). The primers used for amplification of FUT3 were described by Higai (Higai 2006). FUT5 primers were designed as described in (Padro 2011). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (GeneCardsdatabase, NCBI36:X) was used as

an internal control of normalization. Data collection was performed on the ABI Prism 7900HT systems according to the manufacturer's instructions. The primers used in semi-quantitative RT-PCR for FUT3 were 5' ACTGGGATATCATGTCCAACCCTAAGTCAC 3' (F) and 5' GGGC CAGGTCCTTGGGGCTCTGGAAGTCG 3' (R). Primers for FUT5 were reported in (179).

Flow cytometry

Lewis antigens expression was analysed in MKN45 cells and GP220 cells. Cells were trypsinized and $2.5x10^5$ viable cells were assessed for each antibody. Primary antibodies were diluted in PBS-1%BSA and incubated for 30 minutes at 4ºC. The primary antibodies used were: T-174 (Le^a) (180), 2.25Le (Le^b) (181), 57/27 (sLe^a) (182), 19-Ole (H-type 2 (H2)) (181), P12 (Calbiochem) (Le^x), 77/180 (Le^y) (182), and KM93 (Chemicon Int.) (sLe^x). Cells were incubated with the secondary antibody Alexa Fluor 488 (Invitrogen) for 30 minutes at 4ºC. After washing, fluorescent analysis was performed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). At least two independent experiments were performed.

Cell adhesion assays

96 wells microplates were coated with 1mg/ml hyaluronic acid (Sigma) or PBS-BSA1% at 4ºC o/n for Hyaluronic acid binding assays and with recombinant hE-selectin (R&D Systems) at 5µg/ml or PBS-BSA1% at 4ºC for 24h in E-selectin binding assays. Plates were blocked with PBS-BSA 1% at room temperature for 1h. Afterwards 10^5 viable cells were added to the wells and incubated at 37ºC for 1 h. In E-selectin binding assays, cells were pre-incubated with 10µg/ml of KM93 antibody, or PBS-BSA1% for 30 min at 4°C. At that time non adhered cells were removed and wells were washed twice with PBS, adhered cells were

estimated by adding 0.5 mg/mL MTT (Thiazolyl Blue, Sigma-Aldrich) in phenol red-free medium for 2 h at 37ºC. Formazan crystals were solubilised with 50µl of DMSO. Optical density was measured at 550 nm. All the experiments were carried out in triplicates. At least two independent experiments were performed.

Cell lysates and Western blot analysis

Cytoplasmic cell lysates were obtained by lysing the cells in 50 mM Tris pH8, 62.5 mM EDTA and 1% Triton X-100 lysis buffer. Protein extracts were run on 8% SDS-polyacrylamide gels. Separated proteins were blotted onto nitrocellulose membranes (Protran), blocked with PBS-BSA 3% overnight and incubated with primary antibodies CD44 (R&D Systems), β-actin (Sigma) for 1 hour at RT CD44 (R&D Systems) antibody following the manufacture's instructions. Membranes were developed with ECL Western Blotting Substrate.

Proliferation assay

Cells were counted and seeded in 96 wells plate. For MKN45 and GP220 cell lines, $5x10^3$ and 10^4 cells, respectively, were added per well. After 24h, 48h and 72h cells were washed with PBS and viable cells were analysed. 100µl of 0.5mg/ml of MTT diluted in phenol red-free DMEM media was added and incubated for 2h at 37ºC. Formazan crystals were solubilised with 50µl of DMSO and were measured at 550nm. All the experiments were carried out in triplicates. At least three independent experiments were performed.

Wound-healing assay

MKN45 cells were seeded in 6 wells plate, a sterile 10µl pipette tip was used to longitudinally scratch a constant-diameter stripe in the confluent monolayer. The medium and cell debris were discarded and replaced with fresh serum-free DMEM twice. Photographs were taken at

0h, 24h 48h and 72h after wounding by LEICA DMIRB microscope using Leica IM50 image manager software. For statistical analysis, four randomly selected points along the wound were marked, and the horizontal distance between the migrating cells and the initial wound was measured at 24h.

Adhesion of tumour cells to human endothelial cells.

HUVECs were seeded to 96-well plates (1 x 10^4 cells/well) in EBM-2 medium (Clonetics), and allowed to grow to confluence for 2 days. The cells were activated with 20 ng/ml TNF-α (R&D Systems) for 5 h at 37°C. MKN45 cells were incubated with BCECF, AM (calcein) at a concentration of 50µg/ml (Invitrogen) at 37ºC for 30 min. Calcein labelled MKN45 cells were incubate with and without CD44 (10µg/ml) for 30 min at 4ºC. $2.5x10^4$ MKN45 cells were added to HUVEC monolayer well. MKN45 cells were allowed to bind for 40 min. Non-adhered cells were washed three times with PBS and cellular binding was determined by fluorimetry.

Statistical analysis

Statistical significance was established when $p \leq 0.05$. To compare the differences observed in the expression levels of FUT3 and FUT5 and in the levels of Lewis antigens in MKN45 and GP220 cells infected with shRNAs Student's t-test was used. Statistical analysis was performed with SPSS 15.0 (SPSS Inc.).

RESULTS

Expression levels of FUT3 and FUT5 mRNA in MKN45 and GP220 cell lines after infection with shRNAs against FUT3 and FUT5

Two gastric cell lines were selected, with different expression levels of FUT3 and FUT5, and with a different expression pattern of Lewis antigens. In MKN45 cell the expression levels of FUT5 is higher than FUT3 whereas in GP220 cell the expression levels of FUT5 is lower than FUT3 (Figure 17). MKN45 cells have high levels of sLe^a and Le^x, intermediate levels of H-type 2 and sLe^x and low levels of Le^a, Le^b and Le^y. GP220 cells express high levels of Le^y, H-type 2, Le^x, Le^b and Le^a middle levels of sLe^a and low levels of sLe^x.

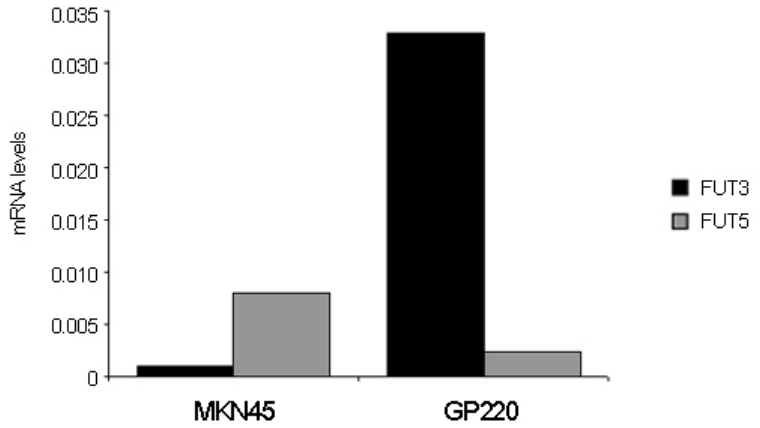


Figure 17. mRNA levels of FUT3 and FUT5 expressed in MKN45 and GP220 cell lines analyzed by qPCR and normalized by HPRT levels.

To analyze the efficiency and specificity of each sequence of shRNAs, the expression levels of FUT3 and FUT5 was analyzed by qPCR in both cell lines after infection of each sequence (data not shown). The

two sequence showing the best specificity and silencing capacities were selected for FUT3 as well as for FUT5. For the FUT3 gene, sequences TRCN0000035864 and TRCN0000035867 were selected. concerning FUT5 sequences TRCN0000035924 and TRCN0000035925 were chosen.

In further experiments we used the combination of TRCN0000035864 and TRCN0000035867 to down-regulate FUT3. The resulting stably infected cells will here be referred to as “shFUT3 cells”. The sequences TRCN0000035924 and TRCN0000035925 served to knock-down FUT5 (“shFUT5 cells”). The four sequences were also used together (“shFUT3/5 cells”).

The FUT3 and FUT5 mRNA levels were analyzed by qPCR in MKN45 and by semi-quantitative PCR in GP220. shFUT3 cells showed decreased levels of FUT3 but not of FUT5. In shFUT5 cells only levels of FUT5 changed. In shFUT3/5 the levels of both FUT3 and FUT5 changed(Figure 18 and 19).

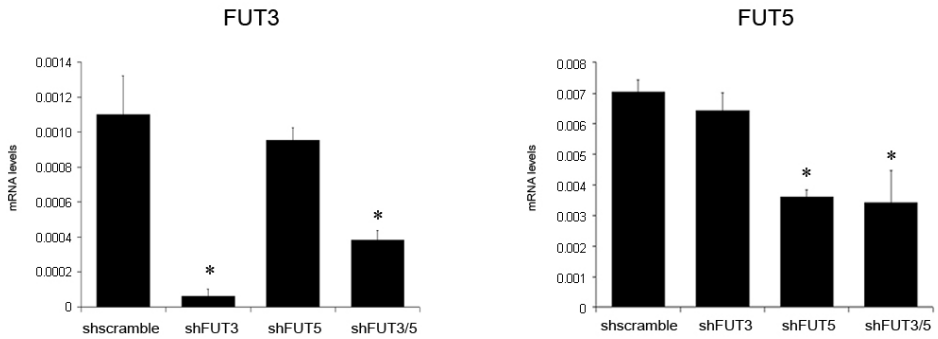


Figure 18. mRNA levels of FUT3 and FUT5 expressed in MKN45 cells infected with shRNAs analyzed by qPCR and normalized by HPRT levels. *$p \leq 0.05$

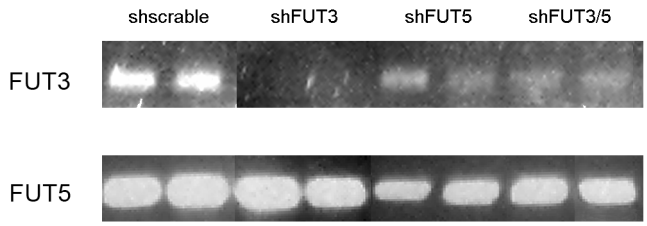


Figure 19. mRNA levels of FUT3 and FUT5 expressed in GP220 cells infected with shRNAs analyzed by semi-quantitative PCR.

Silencing of FUT3 and FUT5 induces changes in Lewis antigens expression pattern on MKN45 and GP220 cells.

The expression of Lewis antigens was evaluated in MKN45 and GP220 cells by flow cytometry after the infection with shscramble, shFUT3, shFUT5 and shFUT3/FUT5, in order to elucidate the implication of these fucosyltransferases on Lewis antigens expression. In MKN45 cells the main changes in the expression pattern of Lewis antigens were detected in FUT5 silenced cells whereas the changes were less pronounced in shFUT3 cells.

In MKN45 cells the down-regulation of shFUT3 induced alterations in expression levels of Lewis antigens. In regard to type II antigens, only sLe^x was reduced (18.17%). Concerning type I antigens Le^a (30.09%) and sLe^a (15.37%) were reduced, although only the reduction of sLe^x and Le^a were statistically significant (Figure 20 and Table 3).

Silencing FUT5 in MKN45 cells induces higher changes in Lewis antigens pattern than down-regulation of FUT3. The main changes were observed in type II antigens, H2 levels were significantly increased (76.79%), and sLe^x levels were significantly reduced (20.29%), while Le^x levels were reduced only an 8%. Since in MKN45 cells the Le^x expression levels are very high, it is very difficult to detect a reduction in percentage of number of cells, so the fluorescence intensity was also analyzed, and regarding fluorescence intensity a statistically significant reduction (45.25%) was detected (Figure 20). In type I antigens only an increase in Le^a (22.95%) and a reduction on sLe^a (8.45%) were observed (Table 3).

In shFUT3/5 MKN45 cells the expression pattern of Lewis antigens was similar than in shFUT5 cells. A statistically significant increase of H2 (78.41%) and a significant reduction of sLe^x (21.66%) were detected. Regarding Le^x levels only a reduction of 4.64% in number of positive cells was observed, but the fluorescence intensity showed a statistically significant reduction (38.99%). In type I Lewis antigens only a reduction of sLe^a (10.02%) was detected (Table 3).

	Le^y	**H2**	**Le^x**	**sLe^x**	**Le^b**	**Le^a**	**sLe^a**
shscramble	12.77	25.18	98.31	44.96	-	18.11	85.29
shFUT3	8.88	26.91	97.32	36.79	-	11.50	72.18
shFUT5	11.43	40.30	88.99	33.89	-	29.34	78.64
shFUT3/5	9.72	44.92	93.70	35.22	-	19.85	76.75

Table 3. Lewis antigens expression levels in MKN45 cells after shscramble, shFUT3, shFUT5 and shFUT3/5 infections. Expressed as a mean of percentage of positive cells.

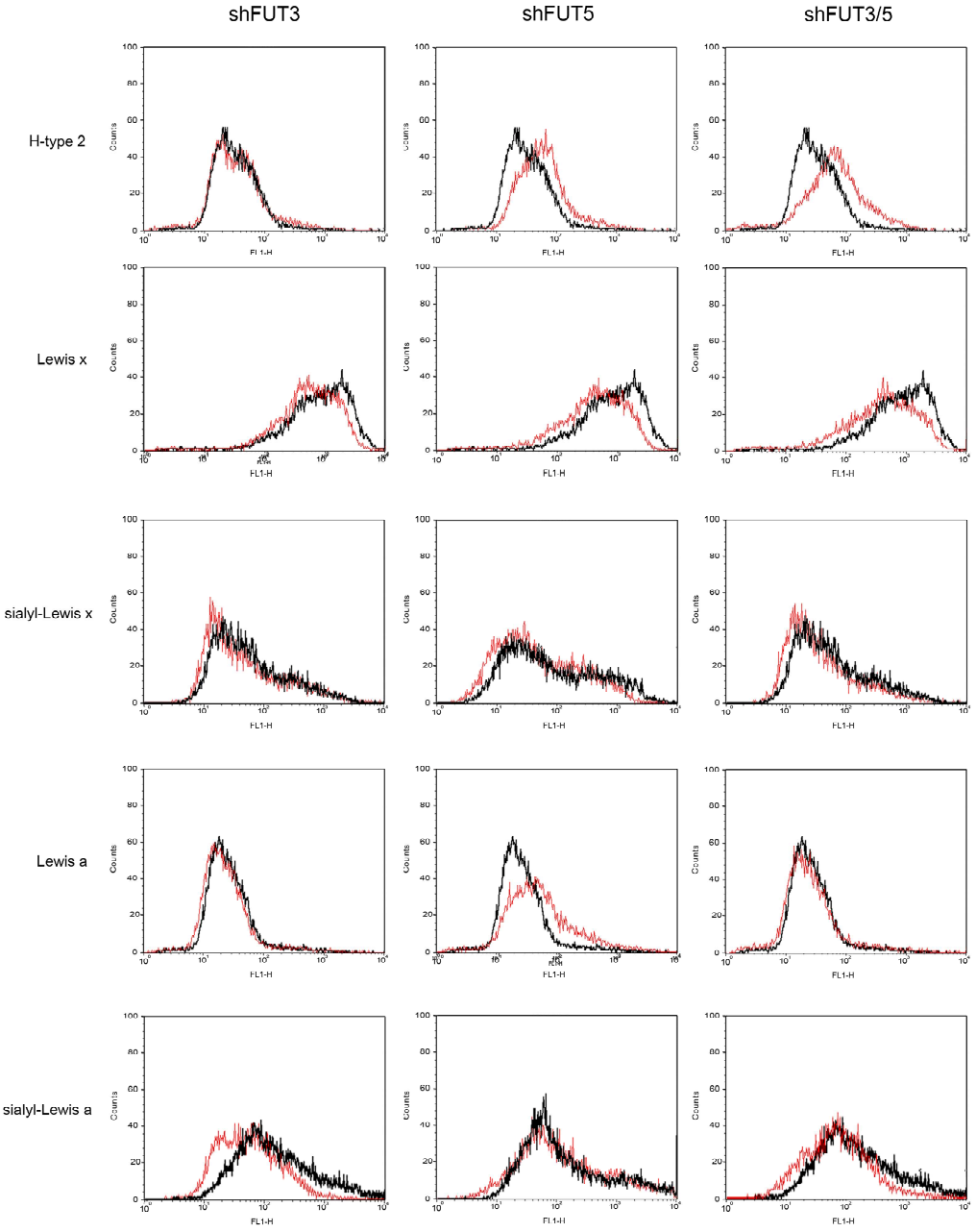


Figure 20. Flow cytometry histograms of Lewis antigens in MKN45 cells infected with shRNAs. The histograms shown are a representative of several experiments. Black lines represent shscramble cells and red lines represent silenced fucosyltransferases cells.

In GP220 cells changes in expression levels of Lewis antigens were smaller than in MKN45 cells. In GP220 cells the most dominant change in Lewis antigens was detected in shFUT3 cells: within type II antigens only Le^y levels decreased (13.48%) whereas all type I Lewis antigens were reduced: Le^b (16.65%), Le^a (10.20%), sLe^a (25.85%). In shFUT5 cells only sLe^a was reduced (25.58%). In shFUT3/5 cells the expression pattern of Lewis antigens was similar than in shFUT3 cells. The expression of the following Lewis antigens was reduced: sLe^a (42.28%), Le^a (5.16%), Le^b (14.74%) and Le^y (9.03%) (Figure 21).

	Le^y	**H2**	**Le^x**	**sLe^x**	**Le^b**	**Le^a**	**sLe^a**
shscramble	80.73	94.83	95.66	4.05	69.45	93.61	18.45
shFUT3	69.84	90.31	94.23	4.59	57.89	84.06	13.68
shFUT5	77.04	92.39	96.20	4.14	71.91	93.70	13.73
shFUT3/5	73.44	91.86	94.98	4.13	59.21	88.78	10.65

Table 2. Lewis antigens expression levels in GP220 cells after shscramble, shFUT3, shFUT5 and shFUT3/5 infections Expressed as a mean of percentage of positive cells.

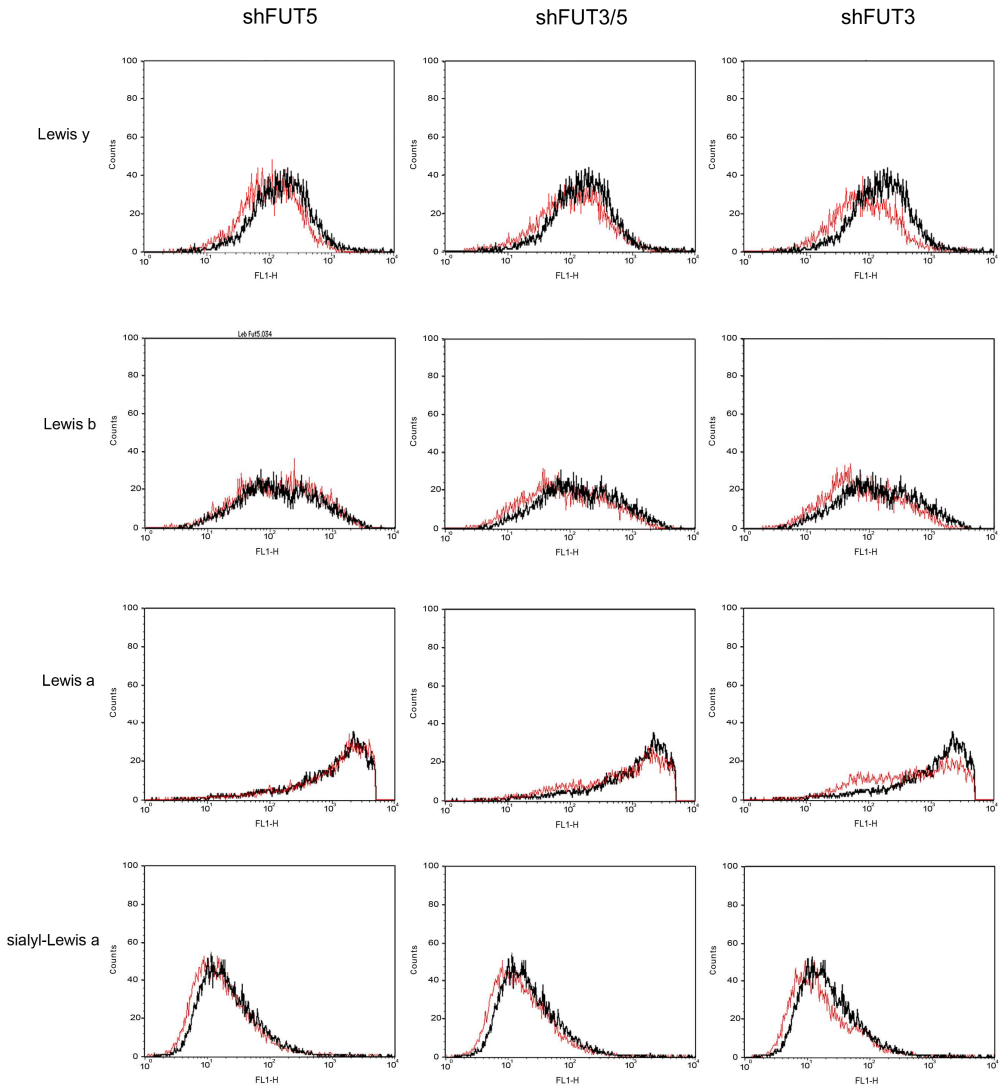


Figure 21. Flow cytometry histograms of Lewis antigens in GP220 cells infected with shRNAs. The histograms shown are a representative of several experiments. Black lines represent shscramble cells and red lines represent silenced fucosyltransferases cells.

Decrease of sLe^x expression levels reduces adhesion of MKN45 cells to E-selectin

In GP220 cells the expression levels of sialyl-Lewis antigens are very low, as a consequence do not present adhesion to E-selectin in *in vitro* assays (data not shown). Therefore, the adhesion assays were performed only in MKN45 cells.

To analyze if the decrease of sialyl-Lewis antigens expression detected in MKN45 cells, when FUT3, FUT5 and FUT3/5 were silenced, was able to induce changes on E-selectin adhesion a E-selectin binding assay was performed. Although the capacity to bind to E-selectin was reduced in shFUT3, shFUT5 and shFUT3/5 cells, only statistically significant decreased levels were detected in shFUT5 and shFUT3/5 cells with a reduction of 42.97% and 42.20% respectively (Figure 22). To elucidate the implication of sLex in E-selectin adhesion, cells were pre-incubated with anti-sLex antibody. The E-selectin binding was reduced 40.57% in shscramble cells, whereas pre-incubation with anti-sLex antibody did not result in a significant difference between shscramble cells and shFUTs cells (Figure 22). These results indicate that the reduction of E-selectin binding in shFUT cells was due to the decrease of sLex expression levels.

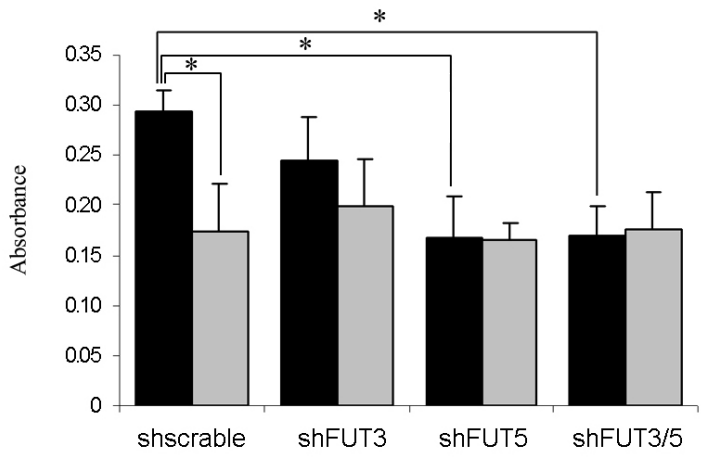


Figure 22. Binding of MKN45 shRNAs cells to E-selectin. Black bars represent no pre-incubated cells. Grey bars represent pre-incubated with anti-sLe^x antibody cells. * $p \leq 0.05$

Adhesion of MKN45 cells to endothelial cells is altered by shFUT3 and shFUT5

To asses if the changes in expression pattern of Lewis antigens in shFUT3, shFUT5 and shFUT3/5 cells were able to reduce adhesion to endothelial cells, the binding of shFUTs cells to a monolayer of HUVEC cells was analyzed. To induce the expression of E-selectin on the surface of HUVEC, cells were stimulated with TNF-α. The capacity of shscramble, shFUT3, shFUT5 and shFUT3/5 MKN45 cells to bind to TNF- α stimulated versus non-stimulated HUVEC cells was tested. The binding was significantly decreased in shFUT3 cells (44.47%), in shFUT5 cells (40.28%), and in shFUT3/5 cells (61.60%) as compared with the scramble control (Figure 23).

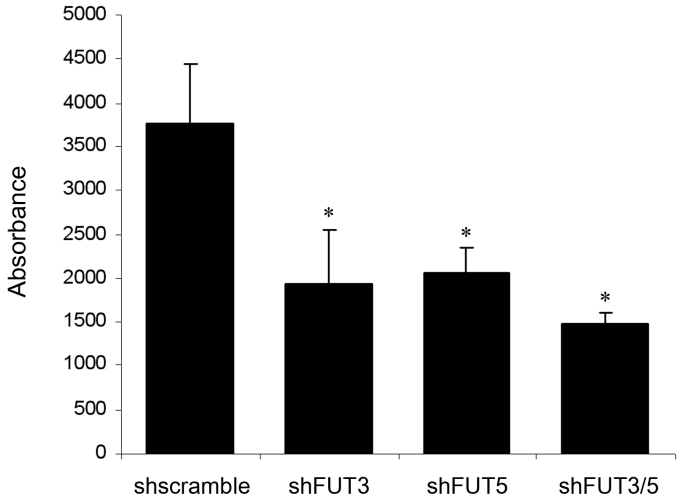


Figure 23. Adhesion levels of MKN45 to human endothelial cells, expressed as the difference between the adhesion to stimulated and non-stimulated HUVEC. * $p \leq 0.05$

CD44 expression levels in shFUT3 and shFUT5 MKN45 cells and hyaluronic acid binding assay

A binding assay was performed, to analyse if the changes in expression of Lewis antigens observed in silenced FUT3 and FUT5 cells can affect the adhesive capacities of MKN45 cells to hyaluronic acid. The main ligand for hyaluronic acid is CD44, and the glycosylation of CD44 has been reported to affect the binding. A significant reduction in the adhesion to hyaluronic acid was observed: in shFUT3 cells (55.93%), shFUT5 cells (64.88%), and shFUT3/5 cells (76.32%) (Figure 24).

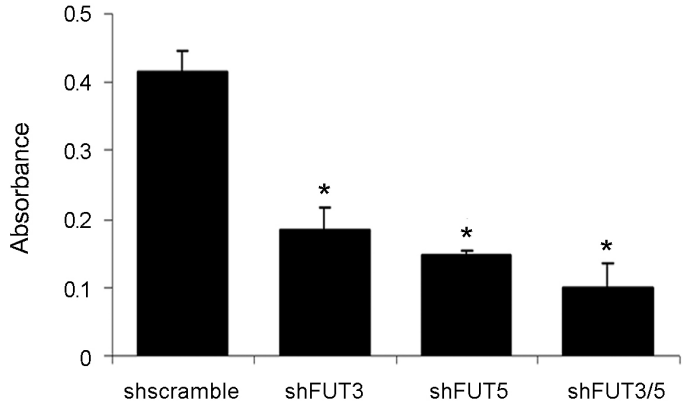


Figure 24 Adhesion to hyaluronic acid of MKN45 cells infected with shscramble, shFUT3, shFUT5 and shFUT3/5. * $p \leq 0.05$

To confirm that the alteration on hyaluronic acid binding was not due to modifications on expression levels of CD44 western blot analysis was performed. No differences in CD44 protein levels were detected between shscramble cells and shFUTs cells (Figure 25).

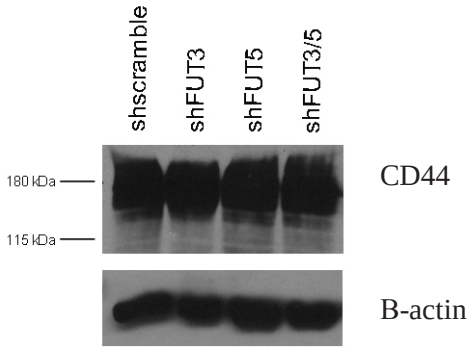
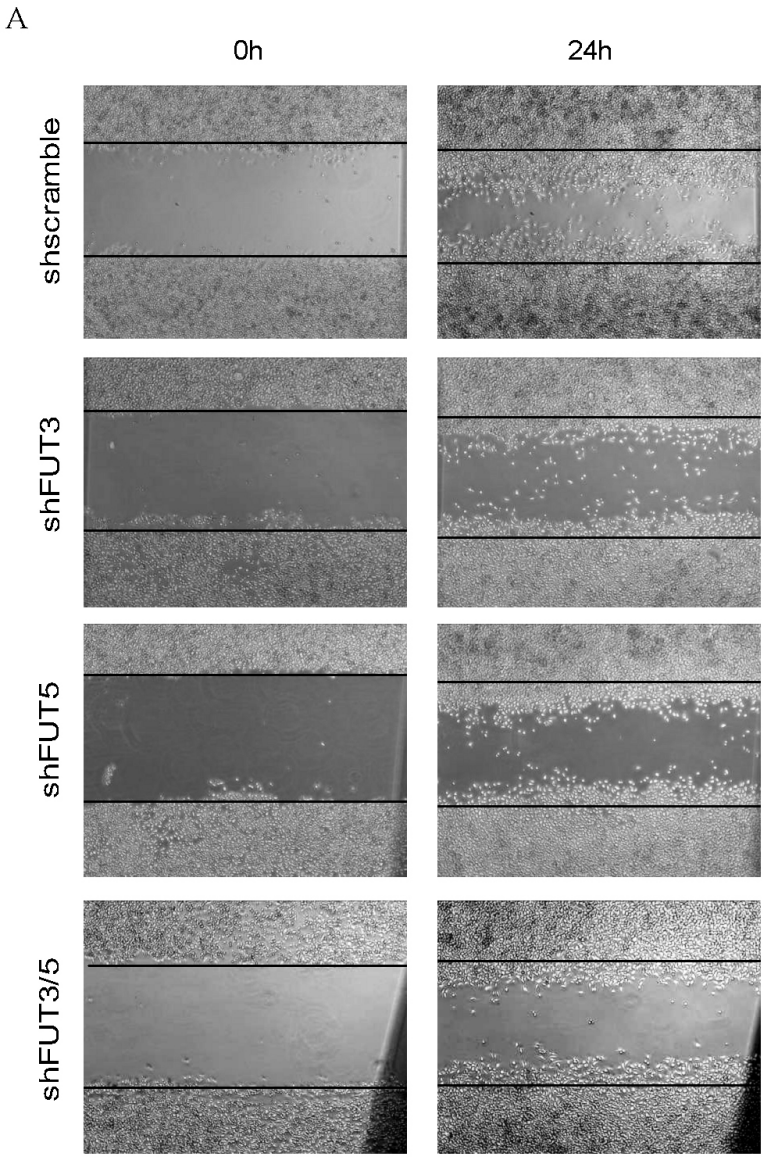


Figure 25. Western blot of CD44 glycoprotein in MKN45 cells infected with shscramble, shFUT3, shFUT5 and shFUT3/5.

Change on Lewis antigens expression levels related with cell migration

To asses if the migratory capacities of MKN45 cells were affected when FUT3 or FUT5 were silenced a wound healing assay was performed. First, to confirm that the infections of MKN45 cells with shscramble and shFUTs were not affecting cell proliferation a MTT assay was performed. No significant differences in proliferation rates were observed between the shscramble and shFUT3, shFUT5 and shFUT3/5 cells (data not shown). In the wound healing assay a reduction on migration abilities of MKN45 cells infected with shFUT3, shFUT5 and shFUT3/5 was detected. Migration was analyzed at 24 h (Figure 26A). The migration capacity in shFUTs cells was reduced compared to shscramble cells: in shFUT3 cells (45.77%) in shFUT5 cells (28.87%) and in shFUT3/5 cells (29.58%) (Figure 26B).


A
0h
24h
shscramble
shFUT3
shFUT5
shFUT3/5

B

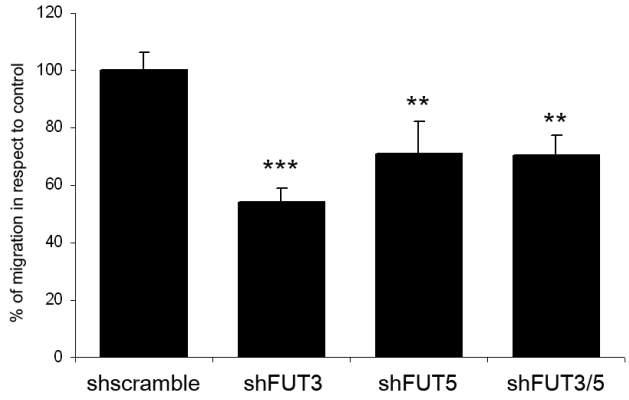


Figure 26. Analysis of cell migration of MKN45 cells infected with shFUT3, shFUT5 and shFUT3/5 by wound healing assay. (A) Wound image at time point 0h and 24h. (B) Quantification of cell migration expressed as percentage of migration in respect to shscramble cells.

DISCUSSION

1. Inhibition of glycosidases by novel iminosugars derivatives

Iminosugars have been postulated in the last decade as a main and potent glycosidases inhibitors, being tested for treatment of different kind of glycosidic disorders diseases (86). Several approaches to increase their inhibitory efficiency have been postulated. The major effort has been focus on chemical structure modifications. The addition of an alkyl chain with different number of C atoms to several sugar analogues (183) has been studied as one of the principal strategies. These alkyled iminosugars obtained have been reported to increases the up taking of the cells, due to the increase on hydrophobicity of the compound given by the *N*-alkyl chain. The hydrophobic chain leads the compound to interact with the cell membrane and facilitated the iminosugar to reach the cytoplasm (184). This membrane interaction in long *N*-alkyl chain can generate plasma membrane disruption due to a detergent-like effect (101). Therefore, higher cellular association and cytotoxicity in the *N*-alkylated-derivatives of the iminosugars have been reported.

In our study, the cytotoxic effects of newly synthesized *N*-alkylated D-fagomine derivatives with *N*-alkyl chain length from 4 C-atoms to 12 C-atoms in eight human cancer cell lines were tested. Results indicated that the cytotoxicity and the associated phenotypic alterations increased as the alkylated chain extends, being the derivative with 12 C-atoms chain the most cytotoxic one. To verify that the effects on cytotoxicity were due to *N*-alkyl chain, another iminosugar, (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol, with *N*-dodecyl chain, was tested. The effects on cytotoxicity were similar than the effects detected in *N*-dodecylfagomine treatments, confirming that the cytotoxic effects were associated with *N*-alkyl chain. Regarding the organ origin of the different cell lines, no differences were detected.

We have detected that the length of the *N*-alkyl chain is also important in the inhibition of the enzymatic activities when cell lysates were treated with the new iminosugars used in our study. Using the D-fagomine derivatives, α-glucosidase inhibition was detected when the cell lysates were incubated with *N*-dodecyl-D-fagomine, whereas no inhibition was found in *N*-butyl-D-fagomine derivative. In this system, these compounds do not alter the activity of other glycosidases. By contrast, previous data indicated that *N*-dodecyl-D-fagomine, was also effective in the inhibition of β-galactosidase from bovine liver (185). One possibility to understand this difference between studies using cell lysates and studies with recombinant enzymes is the fact that MKN45 cell lysates have a high β-galactosidase concentration or activity. To achieve the problem the incubation time was reduced 3 fold, but was not enough. We can conclude that in MKN45 cell lysates *N*-dodecyl-D-fagomine shows a clear inhibition of α-glucosidase, being in agreement with the hydroxyl conformation, as D-fagomine mimics the hydroxyl distribution of glucose, but not galactose molecules (figure 27).

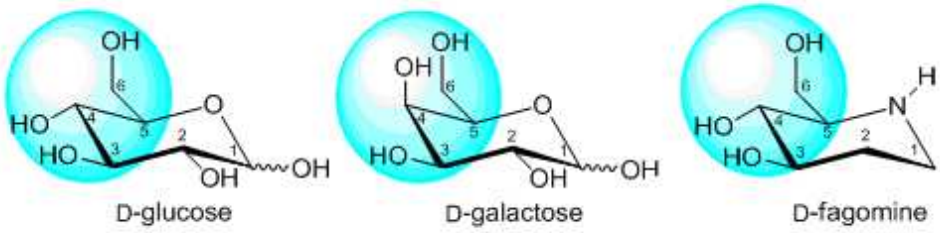


Figure 27. Structure of the iminosugar D-fagomine and the monosacharides D-glucose and D-mannose

The *N*-(2*R*,3*S*,4*R*,5*S*)-1-dodecyl-2-(hydroxymethyl)-5-methyl-pyrrolidine-3,4-diol, a fucose analogue, is able to inhibit the α-L-fucosidase activity in cell lysates whereas no inhibition in α-glucosidase activity was detected. These results correlate with the data obtained using α-L-fucosidase from bovine kidney whereas no effect was observed in the activity of β-D-galactosidase from *Aspergillus oryzae*, α-D-glucosidase from bakers yeast and β-D-glucosidase from sweet almond(Figure 10) (97).

Considering all these data, we can conclude that the N-alkyl chain enhances or facilitates the inhibition, as when the N-alkyl chain is short or not present no inhibition was detected, although the enzyme specificity is given by the polar head of the iminosugar.

The hydrophobic properties of *N*-alkyl chain have been reported to increase the enzymatic inhibition effects of iminosugars by facilitating the uptaking by cell. However, we have performed *in vitro* assays so this would not affect in our assay, but we postulate that the hydrophobicity properties of the *N*-alkyl chain can facilitate the compounds to reach and interact with the active centre of the enzymes (hydrophobic pocket).

2. Regulation of glycosyltransferases and Lewis antigens expression by IL-1β and IL-6.

Fucosyltransferases and sialyltransferases are the enzymes involved in the final steps of Lewis antigens synthesis. The expression of these enzymes determines the Lewis antigens pattern detected in each cell type or tissue. Alterations in Lewis antigens expression have been described in malignant transformation and over-expression of sLe^x in

tumour cells has been correlated with poor prognosis in gastric cancer (186). sLe^x interacts with E-selectin, mediating carcinoma cell – endothelial cell contacts and promoting the extravasation of the tumour cells. (76). We have observed that gastric adenocarcinomas developing metastasis have higher levels of sLe^x, being in agreement with previous studies reporting that sLe^x is over-expressed in gastric cancer and it is associated with metastasis development (186;187).

Clinical studies have described that the presence of chronic inflammation in the digestive tract increases the risk of carcinogenesis (188). Several studies have been published describing the regulation of glycosyltransferases in different cellular models. In hepatocellular carcinoma a increase in the expression levels of FUT6 and ST3GalIV in IL-1β treatments has been reported, affecting the synthesis of sLe^x (189); in colon cancer cells, TNF-α enhances expression levels of FUT4 among several glycosyltransferases (190). In bronchial mucosa explants stimulated with IL-6 and IL-8 increase the expression levels of FUT3, FUT11, ST3GalVI and ST6GalII, modifying the synthesis of sLe^x (191). More recently, the stimulation of the bovine ST6GalII by IL-6 and not by IL-1β and TNF-α, has been reported (192); and in endothelial cells treated with TNF-α have been reported to augment the expression levels of FUT1 leading to enhance the expression of Le^y (193). Regarding gastric data, no studies have been publish. Therefore, we have studied the implication of two inflammatory pathways, NF-kB pathway and JAK/STAT pathway, in the regulation of fucosyltransferases, sialyltransferases and expression pattern of Lewis antigens in MKN45, gastric cancer cell lines, using IL-1β and IL-6 cytokines treatments.

Our results indicate that the fucosyltransferases analyzed in this study can be differentially regulated by two pro-inflammatory cytokines. FucT I and FucT II catalyze the addition of fucose in α-1,2 position,

whereas FucT I is specific for type II precursor chains, FucT II can act in both precursor structures although it acts preferentially in type I precursor chains. In IL-1β treatments, both α1-2 fucosyltransferases are significantly up-regulated whereas in IL-6 treatment only FUT1 is activated. Concerning α1-3/4 fucosyltransferases (FucT III FucT IV and FucT V) only FucT III and FucT V can catalyze the addition of fucose in α1-3 and α1-4 position, even though they do not present the same efficiency to type I and type II structures (73). FucT III and FucT V are drastically down-regulated in IL-1β and IL-6 treatment whereas FucT IV presents in IL-1β treatments an up-regulation at the initial times and a down-regulation at long time treatments and it is not altered under IL-6 stimulus. By contrast, no significant changes in the expression levels of the ST3GalIII and ST3GalIV were detected after the IL-1β and IL-6 stimulation on MKN45 cells.

The regulation patterns detected in pro-inflammatory cytokines treatments seem to be in accordance with the homology and cluster distribution. FUT1 and FUT2 show a similar regulation and both are located in the same chromosome cluster (19q13.3), an exhibit a 67% of homology in the amino acid sequence (Figure 28). FUT3 and FUT5 are also regulated in a similar way and both are located in the same chromosome cluster (19p13.3) with 88% of amino acid sequence homology (194;195). In contrast, FUT4 located in the chromosome 11q21 display less than 45% of amino acids sequence homology with FUT3 and FUT5 (39) (Figure 29). Thus, all the fucosyltransferases of the same cluster respond similarly to the same pro-inflammatory cytokines, indicating that probably the cytokines regulate the whole cluster regulation.

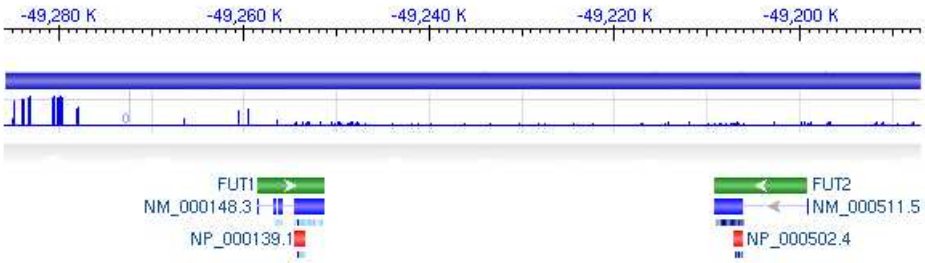


Figure 28. Schematic representation of α1-2 Fucosyltransferases chromosome cluster (FUT1 and FUT2).

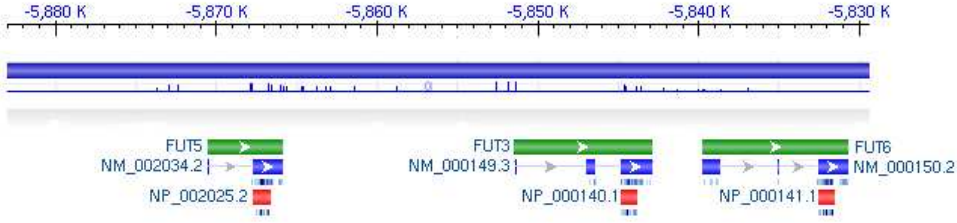


Figure 29. Schematic representation of FUT3, FUT5 and FUT6 chromosome cluster.

We have evaluated the regulation of fucosyltransferase in IL-1β signalling pathway, analysing if the regulation is through NF-kB pathway or through an alternative one. Concerning our results, the up-regulation of FUT1, FUT2 and FUT4 in IL-1β treatments is mediated by NF-kB pathway. Since when the phosphorylation of IκBα is inhibited with panepoxydone treatments, the effects of IL-1β stimulus are block. Whereas the down-regulation of FUT3 and FUT5 it is not mediated by NF-kB pathway, it has to be related with an alternative pathway. Some

alternative pathways that activate other transcription factors as AP-1 have been described (Figure 30) (196).

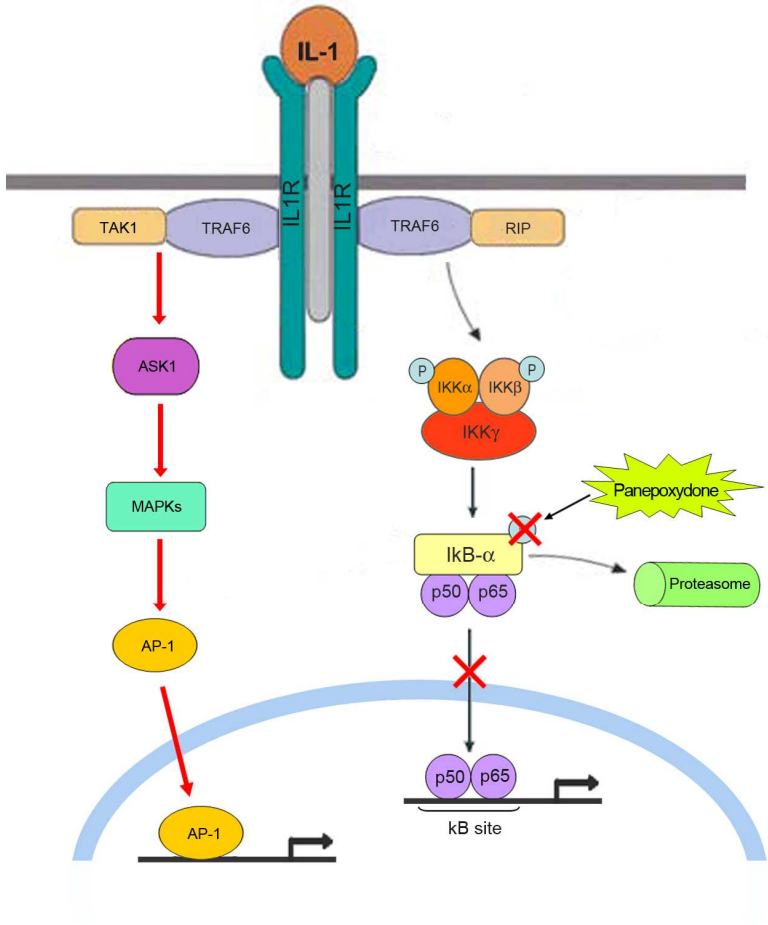


Figure 30. IL-1β signalling pathway. Panepoxydone treatments block the phosphorylation of IκBα inhibiting NF-kB pathway and inducing the increase of alternative pathways as AP-1.

In IL6 treatments, the down-regulation of FUT3 and FUT5 is mediated by gp130/STAT3 signalling pathway because when treatments with AG490 block the STAT3 phosphorylation the levels of those

fucosyltransferases revert. FUT1 levels do not regress in AG490 treatments, indicating that FUT1 is not regulated through gp130/STAT3 signalling pathway. FUT1 regulation could be mediated by JAK/STAT pathway (Figure 31).

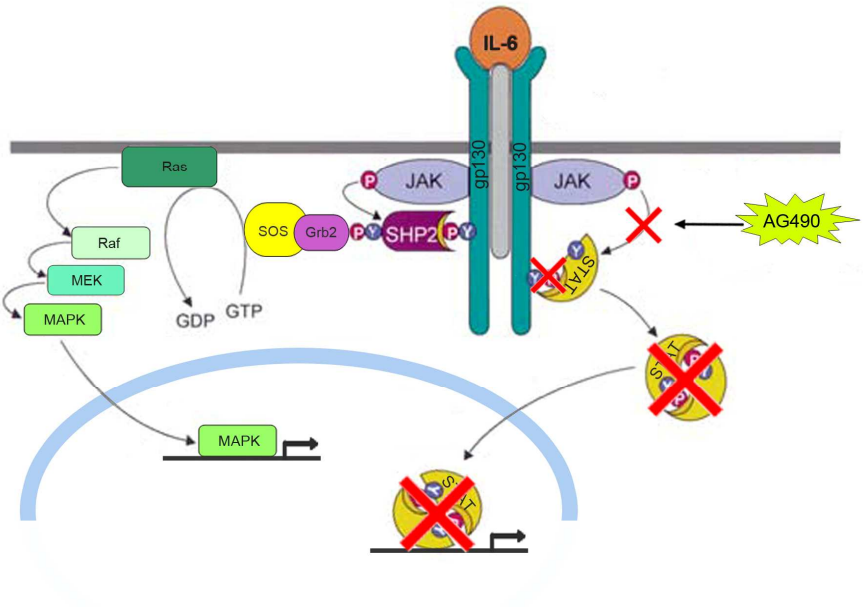


Figure 31. IL-6 signalling pathway. AG490 treatments disrupt the balance between the two pathways increasing the MAPK pathway.

Recent studies have published a cross-talk between NF-kB and pSTAT3 pathway. In epithelial cells an increase in IL-6 productions in IL-1β treatments have been described due to the binding of NF-kB to the IL-6 promoter which induces the production of IL-6 (197). This data is in accordance with no reversion detected in down-regulation of FUT3 and FUT5 detected in panepoxydone treatments as IL-1β treatments could be inducing the production of IL-6.

The increase of FUT1 levels after IL-1β or IL-6 treatments can generate an imbalance in type II Lewis antigens. There are different glycosyltransferases (FUT1, FUT3/5 and ST3Gal III/IV) acting on the precursor structure Galβ1-4GlcNAc, therefore the increase of one glycosyltransferase can disrupt the balance and can generate a change in the Lewis antigens pattern (Figure 32). The increase of FUT1 levels, associated with the decrease of FUT3 and FUT5 levels under IL-1β or IL-6 stimulation, induces a decrease in sialyl-Lewis x levels and an increase of the non-sialylated type 2 structures (H-type 2 and Le^y). These results also agree with recent published data reporting that these α3,4 fucosyltransferases are implicated in the synthesis of the sialylated Lewis antigens in MKN45 cells (198), and it is also in accordance with previously reports where the over expression of FUT1 in colon cancer cells, reduce the levels of sLe^x (143).

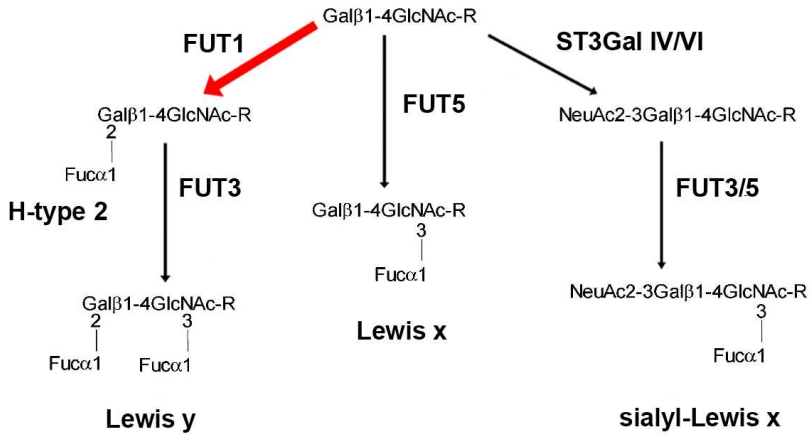


Figure 32. Type II Lewis antigens synthesis. The down-regulation of FUT3 and FUT5 associated with the up-regulation of FUT1, induce the increase of H-type 2 antigen.

Taking all this data in account, we can conclude that inflammatory cytokines are able to regulated glycosyltransferases and consequently modify the expression of carbohydrate structures in tumour cells. Changes on carbohydrates expression pattern can be crucial in the gastric cancer progression.

3. Down-regulation of FUT3 and FUT5 induces alterations in the expression pattern of Lewis Antigens and reduces adhesive capacities of gastric cancer cells

MKN45 and GP220 cell lines with different expression levels of FUT3 and FUT5 were selected. Members of α 1-3/4 fucosyltransferases (FucT III FucT IV and FucT V) have been reported to be altered and involved in malignancy transformation. Among the α1-3/4 fucosyltransferases family only FucT III and FucT V have been described to catalyze the addition of fucose at positions α1-3 and α1-4. While FucT III has a predominant α1-4 activity, FucT V adds fucose with the same efficiency to type I as to type II structures (73). In gastric cancer cells, the knowledge about the FUTs responsibility for synthesis of Lewis antigens is scarce. Therefore, we chose FUT3 and FUT5 to study the expression pattern of Lewis antigens and adhesive capacities of gastric cancer cells.

In regard to Lewis antigens synthesis, in MKN45 cells the down-regulation of FUT3 induced minor changes in the expression pattern of Lewis antigens, among type II only a reduction on sLe^x was detected and among type I a reduction on sLe^a and Le^a was detected. In GP220 the main changes in expression of Lewis antigens induced by down-regulations of FUT3 were detected in type I antigens. In type II, only a reduction in Le^y was found, whereas all type I Lewis antigens were

notably reduced. The down-regulation of FUT5 in MKN45 cells altered principally type II Lewis antigens (sLe^x and Le^x) indicating that FUT5 is involved in their synthesis. In addition, an up-regulation in the expression levels of H-type 2 antigen was detected. H-type 2 antigen is synthesized by FUT1 using type II precursor structure (Galβ1-4GlcNAc) as a substrate. FUT1, FUT5 and sialyltransferases compete for the same substrate and a balance between the three pathways is generated. Subsequently, when FUT5 is down-regulated this balance is disrupted and the precursor available for FUT1 is augmented, increasing the synthesis of H-type 2 (Figure 34). The disruption of this balance in Lewis antigens was previously reported in colon cancer cells where FUT1 over-expression produced an increase of H-type 2 antigens and a decrease of sLe^x (143). In MKN45 cell type I Lewis antigens are lowly expressed, and no important differences were detected in FUT5 down-regulated cells in comparison to the scramble control. In GP220 cells the down-regulation of FUT5 did not change the expression pattern of type II Lewis antigens, sLe^x is not expressed and Le^x, Le^y and H-type 2 did not change. In regards to type I antigens, which are highly expressed in GP220 cells, shFUT5 only induced a slight reduction in expression levels of Le^b, Le^a and sLe^a (Figure 33). In general, FUT5 did not induce significant changes neither of type I nor type II in GP220 cells. This suggests that in GP220 cells FUT5 does not play an important role in synthesis of Lewis antigens.

Analysing the results obtained in GP220 and MKN45 cells, we can conclude that FUT5 synthesize preferably type II Lewis antigens whereas FUT3 synthesize preferably type I Lewis antigens. The enzymatic activities of fucosyltransferases which have been tested using synthetic carbohydrate acceptors are in accordance with our results obtained in gastric cancer cells (73). Thus, although fucosyltransferases have overlapping activities they display preferences in the synthesis of

Lewis antigens. Therefore, depending on which fucosyltransferase is expressed the cells will synthesize mainly type I or type II antigens.

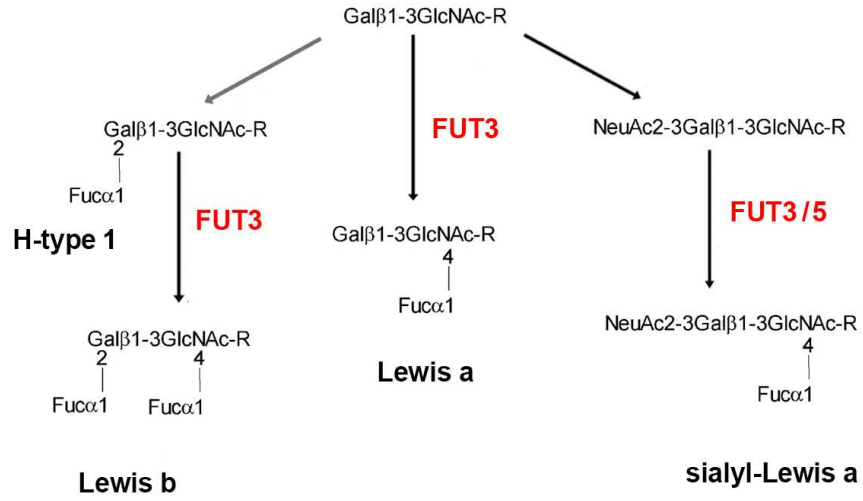


Figure 33. General scheme of type I Lewis antigens summarizing all the data collected in MKN45 and GP220 cell lines.

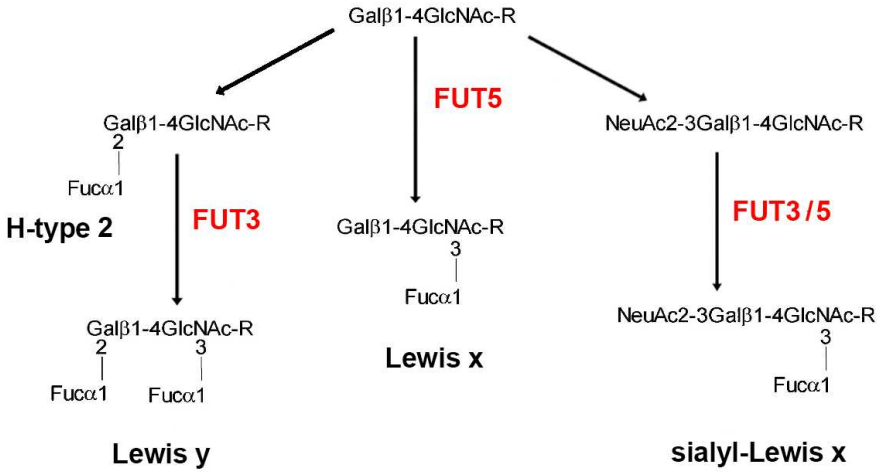


Figure 34.. General scheme of type II Lewis antigens summarizing all the data collected in MKN45 and GP220 cell lines.

Sialyl-Lewis antigens are the major terminal carbohydrate structures that bind selectins and interfere in tumour cell rolling and metastasis (199). It is fundamental to understand the biosynthesis of sLe^a and sLe^x and any mechanism that regulates their synthesis for a further modification of the metastatic phenotype in tumour cells. We have observed that FUT3 and FUT5 are involved in the synthesis of sialyl-Lewis antigens in gastric cell lines. Since sialyl-Lewis antigens levels are very low in GP220, we analyzed if the down-regulation of FUT3 and FUT5 reduced the adhesive and migratory capacities of MKN45 cells.

A reduction in E-selectin adhesion was detected when FUT3, FUT5 and FUT3/5 were down-regulated although only shFUT5 and shFUT3/5 presented a statistically significant decrease. The reduction in the adhesion was linked with the decrease on sLe^x expression levels when the cells were incubated with anti-sLe^x to block the interaction sLe^x-E-selectin. No differences in adhesion between shFUT cells and shscramble cells were detected. The adhesion of MKN45 cells to endothelial cells was analyzed to corroborate these results in a more physiological assay. Endothelial cells were stimulated with TNF-α to induce the synthesis and the expression of E-selectin on their surface. In this assay, a significant reduction of adhesion was detected in all shFUT cells. Considering these data we conclude that down-regulation of FUT3 and FUT5 induces diminution of E-selectin mediated adhesion to endothelial cells.

During inflammatory response and in relation with tumour growth overproduction of hyaluronic acid has been reported (166). In malignant tumours, the presence of hyaluronic acid was detected not only in the stroma but also on the surface of carcinoma cells, and overproduction of hyaluronic acid has been found to be related with tumour growth. Thus, the adhesion to hyaluronic acid is closely related with the metastasis

process. CD44 is the main hyaluronic acid receptor and modifications of the carbohydrate structure of CD44 regulates the capacity of binding hyaluronic acid (163;165). Our results indicate that down-regulation of FUT3, FUT5 and FUT3/5 reduces the adhesion of MKN45 cells to hyaluronic acid. In order to confirm that the alteration of hyaluronic acid binding was not due to changed expression levels of CD44, western blot analysis was performed. No differences in CD44 protein levels between shscramble cells and shFUTs cells were detected.

In summary, we have analyzed the implications of FUT3 and FUT5 in the synthesis of Lewis antigens. The results indicate that down-regulation of FUT3 and FUT5 and the subsequently altered Lewis antigen pattern induce a reduction of the metastatic capacities of tumour cells, reducing the capacity to bind hyaluronic acid and the migratory ability. The reduction of sLe^x expression was essential to decrease the E-selectin mediated adhesion to endothelial cells, an important finding in the extravasation process of tumour cells.

CONCLUSIONS

1. The cytotoxicity of novel iminosugars is determined by the length of the *N*-alkyl chain.

2. The specificity of glycosidase inhibition is given by the polar head of iminosugars.

3. The *N*-alkyl chain of synthetic iminosugars is essential to inhibit glycosidases activity.

4. IL-1β up-regulates FUT1 and FUT2 trough NF-kB signalling pathway and down-regulates FUT3 and FUT5 though an alternative signalling pathway.

5. IL-6 down-regulates FUT3 and FUT5 through p-STAT3 signalling pathway and up-regulates FUT1 through an alternative signalling pathway.

6. IL-1β and IL-6 treatments induce a decrease of sialyl-Lewis x levels and an increase of non-sialylated type II Lewis antigens (H-type 2 and Lewis y).

7. FUT3 and FUT5 silencing with shRNAs reduces expression of sialyl-Lewis antigens.

8. The decrease of sialyl-Lewis x expression reduces the adhesion to endothelial cells though E-selectin interactions.

9. Down-regulation of FUT3 and FUT5 and the subsequently altered Lewis antigen expression pattern induce a reduction in hyaluronic binding capacities and migratory abilities.

Modifications of fucosyltransferases induce alteration of Lewis antigens expression levels which changes adhesive capacities of gastric cancer cells.

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