

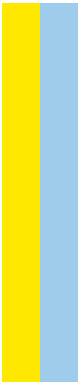


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BIOLOGIA MOLECULAR E CELULAR

Novel strategies targeting cancer-associated glycan biosynthesis as promising cancer drugs

Ana Filipa da Silva Costa

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“To live for the hope of it all”

Para o Vô Valente

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Resumo

O cancro continua a ser um peso na saúde global, com o cancro gástrico (CG) e o cancro colorrectal (CCR) no top dos cancros mais incidentes e mortais em todo o mundo. As terapias atualmente disponíveis para tratamento de cancro consistem em quimioterapia e/ou radioterapia, e terapias dirigidas que são normalmente limitadas a grupos específicos de pacientes, como é o caso de anticorpos monoclonais específicos ou pequenas moléculas. No entanto, ao longo do tempo, estes tratamentos frequentemente apresentam efeitos secundários e resistência. Estes factos salientam a necessidade de explorar novos alvos moleculares, como os glicanos aberrantes presentes em cancro, no desenvolvimento de novas terapias. A glicosilação aberrante, como por exemplo o aumento da sialilação, é uma característica em cancro e é causada principalmente pela desregulação na expressão das enzimas glicosiltransferases. Estes glicanos aberrantes desempenham papéis cruciais em várias características malignas das células tumorais. Por exemplo, a sobreexpressão de Sialyl Lewis X (SLeX) em células tumorais está relacionada com o aumento da agressividade tumoral, como a formação de metástases. Esta tese teve como objetivo elucidar a principal sialiltransferase (ST) envolvida na biossíntese de SLeX e explorar o potencial do mesmo como alvo terapêutico em cancro gastrointestinal (CGI).

Assim, identificámos a ST3GalIV como a principal enzima envolvida na biossíntese de SLeX em células de CGI. Neste trabalho seleccionámos linhas celulares de CGI positivas para SLeX e fizemos um *knockout* (KO) da expressão da enzima ST3GalIV recorrendo à tecnologia de CRISPR/Cas9. O KO desta enzima levou à inibição da expressão de SLeX, com impacto na motilidade celular, o que destaca o papel crítico deste glicano na agressividade das células tumorais. Verificámos também que, numa linha celular em particular, foi necessário um duplo KO das enzimas ST3GalIV e ST3GalVI para inibir completamente a expressão de SLeX. Estes resultados mostram que a ST3GalIV é a enzima principal, embora não exclusiva, na biossíntese de SLeX em CGI, com impacto funcional na motilidade das células tumorais.

Para além do papel essencial da ST3GalIV na biossíntese de SLeX, identificámos duas moléculas como inibidores eficazes da expressão de SLeX em células tumorais, destacando o seu potencial como novas terapias contra o cancro. Através de um *High-throughput screening* (HTS) de mais de 7.800 compostos, identificámos a monensina, um antibiótico ionóforo poliéter, como um inibidor eficaz da expressão de SLeX. A monensina altera a função do Golgi, o que leva a modificações na glicosilação das proteínas. Além disso, a monensina afetou seletivamente as células CGI positivas para SLeX, reduzindo a sua motilidade celular e capacidade invasiva. Através do uso de diferentes modelos *in vivo* mostramos que a monensina consegue reduzir o crescimento e a invasão tumoral. Mostramos também que, modelos de organoides derivados de pacientes com CG e

positivos para SLeX são mais sensíveis à monensina em comparação com organoides derivados da mucosa normal adjacente ao tumor, assim como o seu impacto na expressão de SLeX. O xenotransplante em ratinhos dos organoides derivados de pacientes também mostrou o potencial da monensina como agente anti-tumoral. Em conjunto, estes resultados mostram o potencial da monensina como um novo agente terapêutico contra o cancro.

Nesta tese também explorámos o impacto de análogos fluorados de *N*-acetilgalactosamina (GalNAc) na biossíntese de O-glicanos aberrantes em cancro, nomeadamente SLeX e Sialyl Tn (STn). O tratamento com 4F-GalNAc reduziu significativamente a deteção de O-glicanos aberrantes, assim como levou à alteração da expressão de genes envolvidos na biossíntese de *N*-glicanos e no metabolismo dos açúcares dadores. Consequentemente, o tratamento com 4F-GalNAc reduziu significativa a motilidade e invasão das células tumorais, diminui da ligação à E-selectina, e alterou a ativação de recetores tirosina quinase (RTK) da superfície celular. Além disso, o tratamento com 4F-GalNAc inibiu o crescimento tumoral num modelo de xenotransplante em ratinho. Em geral, estes resultados indicam que o 4F-GalNAc interfere com a biossíntese de O-glicanos aberrantes em cancro, afetando as características malignas das células tumorais, constituindo desta forma uma potencial nova abordagem terapêutica contra o cancro.

Em conclusão, esta tese providencia novos conhecimentos sobre a biossíntese de SLeX em células CGI e a sua associação com a motilidade tumoral. Ao usar o SLeX como alvo, propomos dois potenciais candidatos para desenvolvimento de novas terapias dirigidas que apresentaram resultados promissores em vários modelos *in vitro* e *in vivo* - monensina e 4F-GalNAc. Em conclusão, estes resultados destacam o potencial de usar a glicosilação como alvo para o desenvolvimento de novas terapias eficazes no combate ao cancro.

Abstract

Cancer remains a significant global health burden, with gastric cancer (GC) and colorectal cancer (CRC) ranking among the top in incidence and mortality rates worldwide. The currently available therapies consist of chemo- and/or radiotherapy, and targeted therapies, such as specific antibodies or small drug molecules, that are limited to a specific group of patients. Moreover, over time these treatments often result in side effects and resistance. This underscores the need to explore different and poorly explored targets, such as aberrant glycans in cancer. Aberrant glycosylation, such as increased sialylation, is a feature in cancer and is primarily caused by dysregulation in the expression of glycosyltransferases. Aberrant glycans have been shown to play crucial roles in several malignant features of cancer cells. For instance, the overexpression of Sialyl Lewis X (SLeX) in cancer cells is linked to increased cancer aggressiveness such as metastasis formation. This thesis aimed to elucidate the main sialyltransferase (ST) involved in the biosynthesis of SLeX and explore SLeX potential as a therapeutic target in gastrointestinal cancer (GIC).

We identified ST3GalIV as the primary enzyme involved in the synthesis of SLeX in GIC cells. In this work, we selected SLeX-positive GIC cell lines and knockout (KO) ST3GalIV expression via CRISPR/Cas9 technology. ST3GalIV KO led to the abrogation of SLeX expression and impaired cell motility, highlighting its critical role in cancer cell aggressiveness. We also found that, in particular GIC cell line, a double KO of ST3GalIV and ST3GalVI was required to fully abrogate SLeX expression. These findings portray ST3GalIV as the main, although not exclusive, enzyme contributing to SLeX biosynthesis in GIC cells, with a functional impact on cancer cell motility.

Furthermore, we identified two molecules as potent inhibitors of SLeX expression in cancer cells, highlighting their potential as promising new cancer therapies. Through a High-throughput screening (HTS) of over 7,800 compounds, we identified monensin, a polyether ionophore antibiotic, as a potent inhibitor of SLeX expression. Monensin disrupts Golgi function, leading to alterations in protein glycosylation. Monensin selectively affected SLeX-positive GIC cells, impairing their motility and invasive capacity. Different *in vivo* models showed that monensin reduces tumor cell growth and tumor invasion. In addition, positive SLeX GC derived-organoids were more sensitive to monensin compared to normal mucosa-derived-organoids, with a clear impact on SLeX expression. The xenotransplant of patient-derived organoids in mice (PDOX) also showed monensin potential as an anti-tumor drug. Altogether, these results show the potential of monensin as a novel therapeutical cancer drug.

Additionally, we explored the impact of fluorinated *N*-acetylgalactosamine (GalNAc) analogs on cancer-associated O-glycan biosynthesis, namely SLeX and Sialyl Tn (STn).

4F-GalNAc treatment significantly reduced the detection of cancer-associated O-glycans in cancer cells and led to an alteration in the expression of genes involved in *N*-glycan biosynthesis and sugar donor metabolism. Consequently, 4F-GalNAc treatment led to a significant reduction in cancer cell motility and invasion, a decrease in E-selectin binding, and an alteration in the activation of cell surface receptors tyrosine kinase (RTKs). Furthermore, treatment with 4F-GalNAc inhibited tumor growth in an *in vivo* xenografted mice model. Overall, our results indicate that 4F-GalNAc effectively disrupts the biosynthesis of cancer-associated O-glycans, affecting associated malignant biological features, and constituting a potential novel approach for developing a new cancer therapy.

In conclusion, this thesis provides new insights into SLeX biosynthesis in GIC cells and its association with motility capacities. By targeting SLeX, we propose two potential therapeutic candidates – monensin and 4F-GalNAc – that have shown good results across several models. Hence, these findings highlight the potential of targeting glycosylation to develop novel and effective treatments for cancer.

Abbreviations

5-FU – 5-Fluorouracil

ADC – Antibody-drug conjugate

Asn – Asparagine

BSA – Bovine serum albumin

C1GalT1 – Core 1 GalNAc β 1,3-galactosyltransferase 1

C2GnT – Core 2 β 1,6-*N*-acetylglucosaminyltransferase

CAM – Chick embryo chorioallantoic membrane

CAR-T – T cells expressing chimeric antigen receptors

CC – Colon cancer

CDH1 – Cadherin-1/ E-cadherin

CEA – Carcinoembryonic antigen

CIMP – CpG island methylation

CIN – Chromosomal instability

CMP – Cytidine monophosphate

CMS – Consensus molecular subtypes

COSMC – Core 1 β 3-Gal-T specific molecular chaperone

CRC – Colorectal cancer

CRISPR/Cas9 – Clustered regularly interspaced short palindromic repeats/Cas9

CS – Chondroitin sulfate

DS – Dermatan sulfate

EBV – Epstein-Barr virus

ECM – Extracellular matrix

EGFR – Epidermal growth factor receptor 1

ELLA – Enzyme-linked Lectin assay

EMA – European Medicines Agency

EphB2 – Ephrin type-B receptor 2

ER – Endoplasmic Reticulum

ErbB2 – Human Epidermal Growth Factor receptor 2

ERGIC – ER-Golgi intermediate compartment

EMT – Epithelial-to-Mesenchymal transition

F-MAs – Fluorinated Monosaccharide analogs

FACS – Fluorescence-activated cell sorting

FAP – Familial adenomatous polyposis

FBS – Fetal bovine serum

FDA – United States Food and Drug Administration

FRET – Fluorescence resonance energy transfer

Fuc – Fucose
 FUT – Fucosyltransferase
 GAG – Glycosaminoglycan
 Gal – Galactose
 GalCer – Galactosylceramide
 GalNAc – *N*-Acetylgalactosamine
 GC – Gastric cancer
 GDP – Guanosine diphosphate
 GD – Ganglioside
 GE – Gastroesophageal junction
 GEJ – Gastroesophageal junction adenocarcinoma
 GFP – Green fluorescent protein
 GIC – Gastrointestinal Cancer
 GISTs – Gastrointestinal stromal tumors
 Glc – Glucose
 GlcA – Glucuronic Acid
 GlcCer – Glucosylceramide
 GlcNAc – *N*-Acetylglucosamine
 GMDS – GDP-Man dehydratase
 GO – Gene Ontology
 GPI – Glycosylphosphatidylinositol
 GS – Genomically stable
 GSL – Glycosphingolipid
 GTs- Glycosyltransferases
H. pylori – *Helicobacter pylori*
 HA – Hyaluronan
 HDGC – hereditary diffuse Gastric cancer
 HexNAc – *N*-Acetylhexosamine
 HGFR/ c-MET– Hepatocyte growth factor receptor
 HTS – High-throughput screening
 HS – Heparane Sulfate
 IBD – Inflammatory Bowel Disease
 IdoA – Iduronic Acid
 IGF-I – Insulin-like growth factor 1
 IP – Intraperitoneal
 IT – Immunotoxin
 KEGG – Kyoto Encyclopedia of Genes and Genomes

KO – Knockout
 KS – Keratan Sulfate
 LacNAc – *N*-acetyllactosamine
 LeA – Lewis A
 LeX – Lewis X
 LUC – Luciferase
 mAb – Monoclonal antibody
 Man – Mannose
 MGAT5 – *N*-acetylglucosaminyltransferase V
 MSI – Microsatellite instability
 MW – Molecular weight
 N – Nitrogen
 Neu5Ac – *N*-acetylneuraminic acid
 O – Oxygen
 OGT – Oligosaccharyltransferase
 ON – Overnight
 PD-L1 – Programmed death ligand 1
 PDO – Patient-Derived Organoid
 PDOX – Patient-Derived Organoid Xenograft
 PNGase F – Peptide *N*-glycosidase F
 ppGalNAcT – Polypeptide GalNAc transferase
 PTM – Post-translational modification
 RC – Rectal cancer
 RFLP – Restriction fragment length polymorphism
 RNAseq – RNA Sequencing
 RTK – Receptor tyrosine kinase
 Ser – Serine
 SLeA – Sialyl Lewis A
 SLeX – Sialyl Lewis X
 SCNA – Somatic copy number
 ST3GalIII – β -galactoside α 2,3-sialyltransferase 3
 ST3GalIV – β -galactoside α 2,3-sialyltransferase 4
 ST3GalVI – β -galactoside α 2,3-sialyltransferase 6
 ST6GalI – β -galactoside α 2,6-sialyltransferase 1
 ST6GalNAcI – α -*N*-acetylgalactosaminide α -2,6-sialyltransferase 1
 ST - Sialyltransferases
 STn – Sialyl Tn

TCGA – The Cancer Genome Atlas

Thr – Threonine

TIDE – Tracking of Indels by Decomposition

TPS – Tissue polypeptide specific antigen

VEGF – Vascular endothelial growth factor

WHO – World Health Organization

WT – Wild-type

UDP – uridine diphosphate

Xyl – Xylose.

Table of Contents

CHAPTER 1	1
1. Cancer	2
1.1. Gastrointestinal cancers	3
1.1.1. Gastric cancer	3
1.1.1.1. Classification and carcinogenesis	4
1.1.1.2. Diagnosis and treatment	7
1.1.2. Colorectal cancer	9
1.1.2.1. Classification and carcinogenesis	10
1.1.2.2. Diagnosis and treatment	13
2. Glycosylation in health and cancer	14
2.1. Different types of glycosylation	16
2.2. Glycosylation in cancer	18
2.2.1. The Lewis antigens and the role of SLeX in cancer	20
2.2.2. Role of truncated O-Glycans in cancer	24
3. Strategies for targeting cancer-associated glycans as promising future therapies	25
3.1. High Throughput Screening targeting glycosylation	27
3.2. Fluorinated Monosaccharide Analogs	29
4. Aims and thesis outline	35
5. References:	37
CHAPTER 2	53
ST3GALIV DRIVES SLEX BIOSYNTHESIS IN GASTROINTESTINAL CANCER CELLS AND ASSOCIATES WITH CANCER CELL MOTILITY	53
CHAPTER 3	79
MONENSIN AS A POTENTIAL DRUG FOR TREATMENT OF SLEX-POSITIVE TUMORS	79
CHAPTER 4	131
4F-GALNAC IS A GENERAL INHIBITOR OF CANCER-ASSOCIATED O-GLYCANS IN GASTROINTESTINAL TUMORS	131
CHAPTER 5	169
General Discussion	170
Final Remarks and Future Perspectives	178
References	179
APPENDIX I	187

NOVEL ANTICANCER DRUG DISCOVERY EFFORTS TARGETING GLYCOSYLATION: THE EMERGENCE OF FLUORINATED MONOSACCHARIDES ANALOGS	187
APPENDIX II	199
Scientific Outputs.....	200

Chapter 1

General Introduction Aims and thesis outline

THIS CHAPTER CONTAINS PARTS OF THE FOLLOWING LITERATURE REVIEW:

Costa AF, Teixeira, A, Reis. CA, Gomes, C. Novel anticancer drug discovery efforts targeting glycosylation: the emergence of fluorinated monosaccharides analogs. **Expert Opin Drug Discov.** 2025 Jan:1-11 (Appendix I)

General Introduction

1. Cancer

Cancer is a major public health problem and represents one of the largest economic burdens, with an estimated 20 million new cases and 9.7 million associated deaths worldwide in the year 2022. In the top five of most diagnosed cancers, we can find lung cancer as the most diagnosed worldwide, followed by breast (11.6%), colorectal (9.6%), prostate (7.3%), and gastric (4.9%). Regarding mortality, lung cancer is also the first most deadly tumor (18.7%), followed by colorectal (9.3%), liver (7.8%), breast (6.9%), and gastric (6.8%) cancers (**Fig 1**) [1].

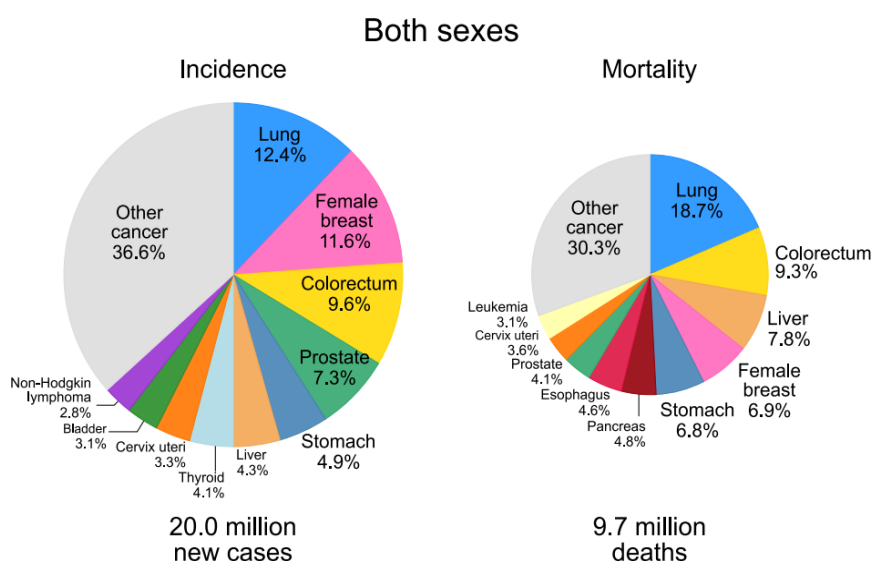


Figure 1: Estimative of cancer incidence and mortality for both sexes in 2022. From [1].

According to the World Health Organization (WHO), the definition of cancer, also termed neoplasm or malignant tumor, is a large group of genetic diseases when abnormal cells grow uncontrollably and go beyond their tissue boundaries. If not controlled, these abnormal cells break away from the original (primary) tumor/ site, travel through the bloodstream or lymph system, and form a new tumor in other organs or tissues of the body. This process is called metastization and is a major cause of death in cancer patients [2].

Cancer development and progression involve different molecular alterations in cells. The cells develop capabilities for sustaining proliferative signaling, evading growth suppressors, resisting to cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming cellular metabolism, promoting inflammation, avoiding immune destruction, and having genome instability with associated mutation. These characteristics are the hallmarks of cancer [3]. However, there is an ongoing debate among specialists as these established and accepted hallmark traits alone fail to capture the complexities of cancer pathogenesis. Thus, in 2022, four new hallmarks

were added to define better characteristics of tumors: nonmutational epigenetic reprogramming, polymorphic microbiomes, senescent cells, and unlocking phenotypic plasticity [4] (**Fig 2**).

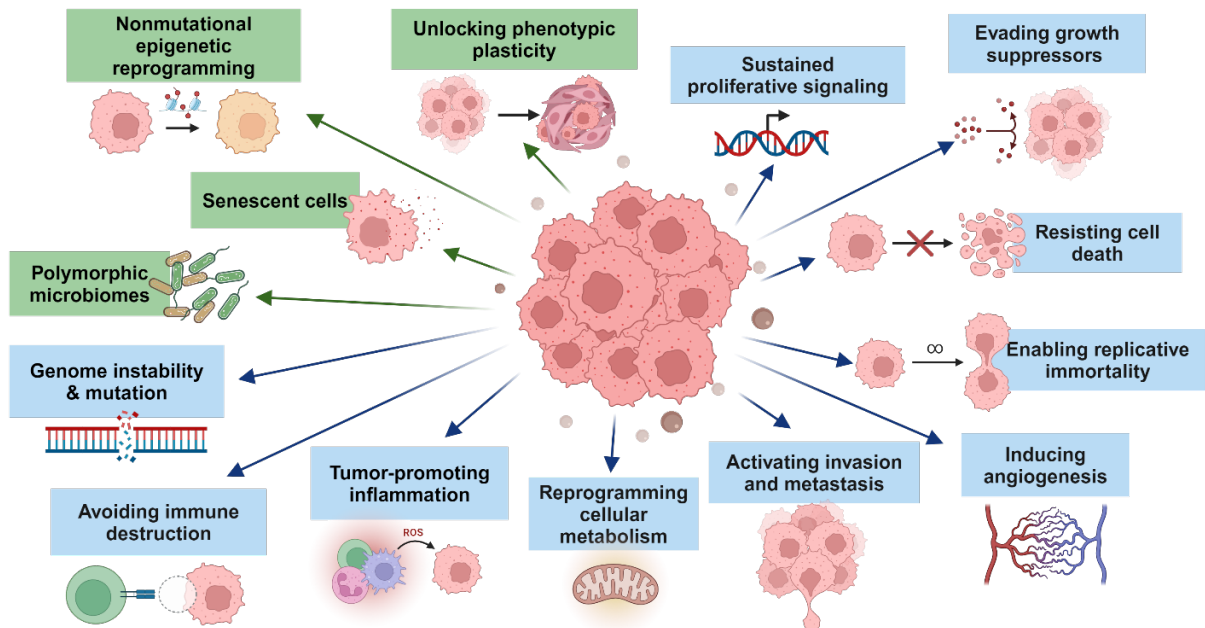


Figure 2: The updated Hallmarks of cancer. Hallmarks of cancer proposed since 2011 are represented in blue, while the newly proposed hallmarks introduced in 2022 are represented in green. Image created with BioRender.com.

1.1. Gastrointestinal cancers

Gastrointestinal cancers (GIC) encompass a highly heterogeneous group of diseases affecting the gastrointestinal tract. This group includes gastric, colorectal, pancreas, esophagus, anus, gallbladder, liver, and bile duct cancers. Given the wide range of organs and tissue, GIC exhibits a diversity in etiology and clinical management [5]. Therefore, in this thesis, we will focus on the more diagnosed and deadly cancers: gastric and colorectal cancers.

1.1.1. Gastric cancer

Gastric cancer (GC) ranks fifth position in terms of diagnosis and cancer-related deaths worldwide. In fact, in 2022, there were over 968,000 new cases of GC and nearly 660,000 deaths in the world. The incidence rates are highest in Eastern Asia alongside Eastern Europe and South America, and lowest across the African continent. More than 50% of the new cases occur in developing countries, and the incidence in men is more than twice as high as in women [1].

Despite these numbers, GC trends have been steadily declining over the last half-century in most populations. This could be mainly attributable to the increased investment

in prevention which includes early diagnostic, routine screening with increased endoscopy exams that enable the diagnosis and treatment of precancerous cases and early-stage cancers [6]. Other reasons are better storage of foods and a decreased prevalence of *Helicobacter pylori* (*H. pylori*) infection [1,7]. However, recent studies have pointed to a rising of new cases among younger people, with an increase in autoimmune gastritis and dysbiosis of the gastric microbiome which is becoming something worrying for society [1,7].

A large majority of GC cases can be attributed to pathogenic infections. The most common pathogen associated with GC development is *H. pylori*. *H. pylori*, a gram-negative bacterium, is one of the most prevalent bacterial infections worldwide, affecting nearly half of the world's total population, and it is classified as a class I carcinogen. The infection leads to a stepwise pattern of disease progression in a small percentage of the infected population, where the chronic inflammation can evolve to gastric atrophy, intestinal metaplasia and ultimately carcinoma. However, if eradication programs are applied and effective in the early stage of the disease progression, the gastric lesions can be reversed [8-10]. The Epstein Barr virus (EBV) has also been shown to influence GC progression in around 10% of GC cases. This DNA virus belongs to the herpes family and induces tumor growth by incorporating virus DNA fragments into the genome of dividing cells, although the mechanism of viral introduction and subsequent infection into a human cell is not clear yet [8,10].

Other risk factors for GC include alcohol consumption, smoking, and poor dietary habits such as high intake of salt, low consumption of fruit and vegetables, high consumption of processed meat, and grilled or barbecued meat [8,11].

GC also could have an associated hereditary background. Mutations in the E-cadherin (CDH1) gene are present in 3% of the GC cases. This type of GC is referred to as hereditary diffuse GC (HDGC) and most of the patients are diagnosed before the age of 40 [10,12].

1.1.1.1. Classification and carcinogenesis

The most common type of GC, which comprises close to 95% of the cases, is stomach adenocarcinoma, which starts in the mucus-producing cells of the stomach lining. Other, less common types include primary gastric lymphoma, gastrointestinal stromal tumors (GISTs), gastroesophageal junction adenocarcinoma (GEJ), and neuroendocrine tumors. Histologically, focusing on adenocarcinomas, different systems have been proposed to stratify GC patients over the years. The most used are the WHO classification, where GC is divided into four subtypes, and the Lauren classification, with two major subtypes and an indeterminate type [7].

The WHO subtypes include the tubular, papillary, mucinous, and poorly cohesive, as well as uncommon histologic variants [13]. This system is vastly used and is very useful, however, the Lauren classification system is the most used system of classification, nowadays. It was developed in the 60s and uses structural cellular components of the disease to separate patients into two main subtypes: well-differentiated or intestinal, and poorly differentiated or diffuse (**Fig 3**), and an indeterminate or mixed type subtype [14,15]. The intestinal type encompassed approximately 54% of the cases and is associated with intestinal metaplasia and *H. pylori* infection [16]. It is commonly found in individuals of an older age (> 50 years) [17]. Around 32% of cases correspond to the diffuse subtype and, usually, have poor survival statistics and are commonly found in younger patients, predominately in women [16,18]. The indeterminate or mixed subtype is present within a much smaller subset of patients, around 14% of the cases, and usually occurs in males, and it is known to be highly invasive and metastatic [19].

The cascade of oncogenic events that preceded the development of GC is also significantly different between subtypes. Long-lasting infection with *H. pylori* triggers the cascade behind the intestinal-type malignant transformation. The first phase, gastritis, is characterized by increased infiltration of the lamina propria with mononuclear leukocytes (chronic inflammation) and polymorphonuclear neutrophils (acute inflammation). A prolonged inflammatory process results in the loss of normal glandular tissue called atrophic gastritis, which can eventually evolve to intestinal metaplasia. This represents a drastic phenotypic change from the normal epithelial cell of the gastric mucosa to an intestinal phenotype and is an advanced stage of atrophy. When the architecture of the tissue no longer maintains well-organized glands and presents neoplastic lesions, it reaches a phase of dysplasia that later evolves into an intestinal-type carcinoma (**Fig 3**) [20].

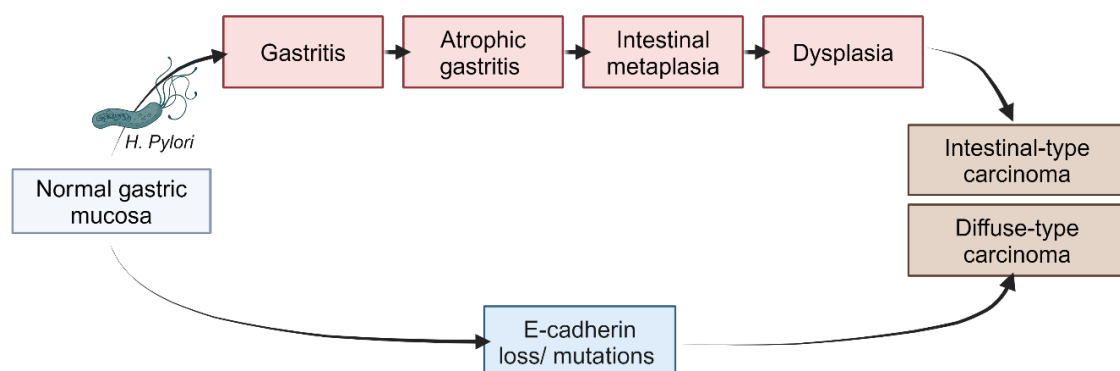
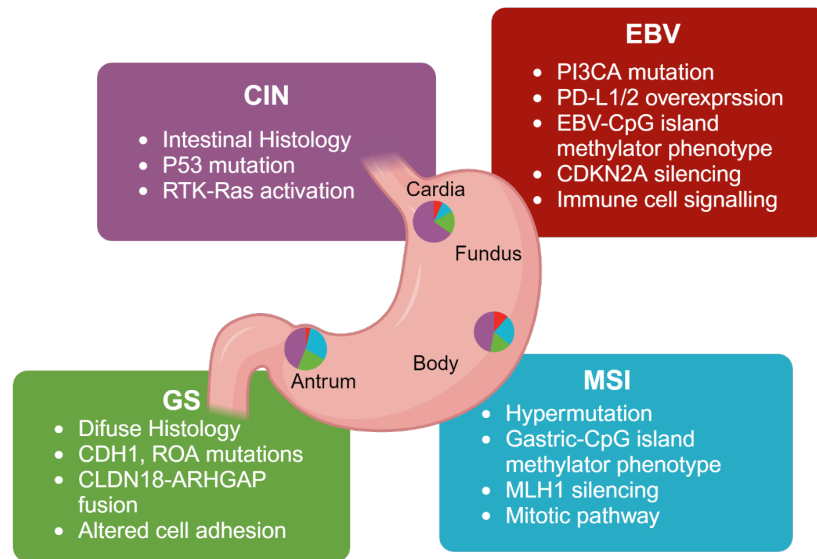


Figure 3: Carcinogenesis of intestinal and diffuse type gastric carcinoma, according to Lauren's classification. Intestinal-type gastric carcinoma results from a cascade of events that starts with an infection by the bacterium *H. pylori* that can colonize the stomach and trigger gastritis that eventually evolves to atrophic gastritis and then to intestinal metaplasia. Intestinal metaplasia progresses to dysplasia, a premalignant condition characterized by disturbed differentiation and growth of epithelial cells leading to an intestinal-type carcinoma. The diffuse type of gastric cancer arises spontaneously and is often characterized by mutations/ loss of function in E-cadherin. Image created with BioRender.com.

Contrariwise, in the development of diffuse-type GC the major molecular alteration is E-cadherin dysregulation/dysfunction. In fact, E-cadherin mutations were described in 50%-80% of diffuse-type GC, contrary to intestinal-type GC (**Fig 3**) [21]. This type of tumor does not involve a pre-malignant lesion. It consists of poorly adhesive, infiltrative cells that typically invade the submucosa at an early phase where malignant cells often spread to the upper layers of the stomach [22]. It is also characterized by the presence of signet ring cells [23].

A



B

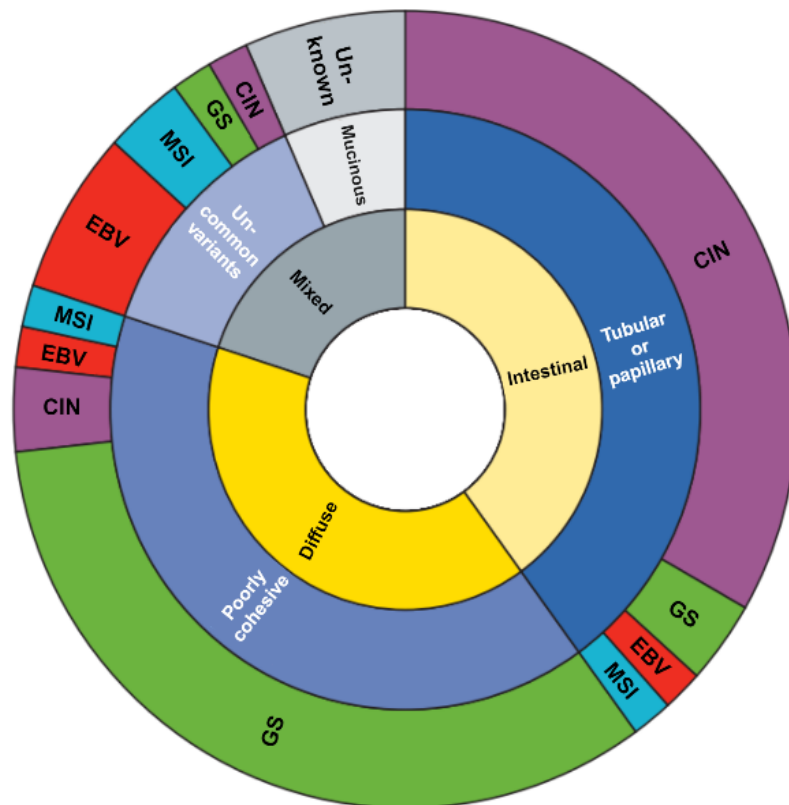


Figure 4: Molecular classification system of gastric carcinoma, as proposed by The Cancer Genome Atlas (TCGA) project. A) Specific oncogenic pathways and molecular features of the four subtypes: CIN (purple), GS (Green), EBV (Red) and MSI (blue). Boxes show molecular features of each subtype and histograms depicting its incidence within the stomach compartments (cardia, fundus, body and antrum). Image created with BioRender.com. Adapted from [24]. **B)** Graphical depiction of TCGA molecular classification (outer circle) overlapping with the existing morphological classifications (Lauren classification – inner circle, WHO classification – Middle circle). Adapted from [6]

CIN – chromosomal unstable. GS – genomically unstable. MSI – microsatellite instable. EBV - Epstein-Barr virus-positive.

Unfortunately, the high degree of histopathological heterogeneity in GC has limited the clinical utility of the Lauren classification system. To address this, in 2014, The Cancer Genome Atlas (TCGA) research network conducted a comprehensive genome-wide analysis and proposed a new stratification of GC patients [24]. This stratification divides patients into four distinct molecular subtypes, each characterized by specific oncogenic pathways and molecular features: 1) EBV-positive tumors which are characterized by PIK3CA mutations and PD-L1/2 amplifications; 2) Microsatellite unstable tumors (MSI), that exhibit high mutation rates with particular impact on key signaling oncogenic proteins, including epidermal growth factor receptor 1 (EGFR), 2 (HER2) and 3 (HER3); 3) Genomically stable (GS) tumors, mainly of the histologically diffuse subtype that presents mutations in genes codifying for E-cadherin (CDH1) and the RHO-family GTPase-activating proteins, and 4) Tumors with intestinal histology containing chromosomal instability (CIN), presenting marked aneuploidy, TP53 mutations and amplifications of Ras proteins and cancer-relevant targetable receptor tyrosine kinases (RTKs) (**Fig 4A**) [24,25]. This proposed genomic classification system overlaps substantially with existing histological classifications (**Fig 4B**) [7].

1.1.1.2. Diagnosis and treatment

The overall survival for GC is around 25% worldwide. The main reason for the low survival rates is that most cases are diagnosed in the later stage of the disease which is characterized by the presence of metastases, high intratumor heterogeneity, and chemotherapeutic resistance. This is mainly due to the fact that in early stages, GC patients are often asymptomatic, and, consequently, when patients present symptoms, the disease has already developed to advanced stages [10].

The most common symptoms associated with GC are indigestion, poor appetite or early satiety, weight loss, and abdominal pain. Difficulty in swallowing or regurgitation might also be a symptom of cancer localized at the gastroesophageal junction. Anemia might be present in bleeding cancers. Normally, if these symptoms are present during diagnosis, the

disease is often in an advance stage [7]. The diagnosis is achieved by endoscopic examination and biopsies are taken for histological confirmation. Clinical staging determines whether the treatment approach will be curative or palliative [7,26].

Prevention, early detection, and treatment of GC at the early stage remain the most effective strategies for improving patient outcomes. Unfortunately, there are no specific serological assays for the certain detection of GC, as the currently approved tumor biomarkers lack the sensitivity and specificity needed for safe use as early diagnostic tools. However, some circulating biomarkers are valuable for monitoring disease progression and assessing patients' therapeutic responses. So far, the three most used serological biomarkers for GC are CA 19.9, CA 72.4 and CEA [27-30]. The CA 19.9 serological assay measures the glycan structure Sialyl Lewis A (SLeA), frequently upregulated in GC. However, this biomarker can also be found in benign diseases and, therefore is used for the clinical monitoring of disease relapse and patient response to therapy [30,31]. The CA 72.4 immunological assay detects the Sialyl Tn (STn) glycan antigen, which is found at abnormally elevated levels in serum. It is used as a prognostic factor of tumor aggressiveness, disease recurrence, and poor outcome of GC patients [30,32]. High levels of the highly glycosylated carcinoembryonic antigen (CEA) can be detected in most patients, however, its applicability as an early diagnostic method for GC is poor, due to sensitivity limitations at early-stage disease [30,31]. Consequently, this marker is used mainly to predict peritoneal recurrence following curative gastrectomy [33].

For early-stage GC, the most common approach is surgical tumor resection rather than systemic chemotherapy [7,10]. The type of surgery depends on the location of the tumor and the degree of invasion in neighbors' tissues, but in general, it includes endoscopic mucosal resection, distal esophagectomy, and subtotal or total gastrectomy [10]. The addition of chemotherapy either pre-, post-, or perioperatively increases the chance for curative resection, since it helps to eliminate early microscopic spread, and allows a better response assessment of treatment [10]. The European Society for Medical Oncology treatment guidelines for GC gives a strong recommendation for perioperative chemotherapy with a combination of platinum (cisplatin, carboplatin, and oxaliplatin) and fluoropyrimidine (capecitabine, floxuridine, and 5-fluorouracil (5-FU)) for patients with stage 1B or greater resectable GC [7]. For inoperable GC, i.e., locally advanced unresectable or metastatic GC, chemotherapy improves patients' survival and quality of life [7,34]. Patients with advanced GC who are treated with combination chemotherapy have a median overall survival of around 1 year compared with 3–4 months when treated with supportive care alone [7].

Amplification of the *HER2* (also known as *ERBB2*) oncogene and overexpression of the HER2 protein occur in approximately 17–20% of GC patients and is more common in the intestinal subtype [35]. For patients with inoperable GC with overexpression of HER2,

the use of anti-HER2 antibody trastuzumab (Herceptin®), combined with fluoropyrimidine and platinum chemotherapy improved the overall survival compared with patients treated with chemotherapy alone [36]. Unfortunately, GC patients undergoing trastuzumab-based therapy showed intrinsic or acquired resistance to the applied treatment [37].

Two novel monoclonal antibodies, Ramucirumab (Cyramza®), targeting a vascular endothelial growth factor receptor 2 (VEGFR-2) which affects angiogenesis and is used in combination with paclitaxel [38,39], or the Pembrolizumab (Keytruda®), targeting programmed cell death protein 1 (PD-L1), are currently used as a second and a third line of treatment. Pembrolizumab is also used in MSI type GC tumors as a second line of treatment [40].

1.1.2. Colorectal cancer

In clinical practice and research, colon cancer (CC) and rectal cancer (RC) are often grouped as colorectal cancer (CRC). This classification is based on three main factors: 1) both cancers develop in the large intestine, which is considered a single organ, 2) they share similar anatomical and histological structures, and 3) the colorectal tract has common functions, including stool concentration, fluid resorption, and excretion. CC corresponds to 72% of all CRC cases while RC corresponds to 28%. Nevertheless, incidence statistics typically combine them, as described in the next paragraph [41].

CRC is the third most commonly diagnosed cancer, with 1.9 million new cases reported in 2022. New cases occur across the globe, however, around 55 % of cases are diagnosed in developed countries. Places with the highest incidence rates include Australia, New Zealand, Europe and Northern America. Conversely, incidence rates are low in Africa, South-Central Asia and Central America. Fortunately, due to the widespread use of colonoscopy screening, the incidence within these countries has been decreasing in the last years, however, the incidence among younger adults is increasing which is worrisome [1]. CRC is also the second deadliest, responsible for 904,000 deaths that same year. This means that one in every ten deaths due to cancer worldwide is attributed to CRC. Furthermore, CRC is the leading cause of cancer in men and second in women younger than 50 years old [42].

Approximately 60–65% of CRC cases happen sporadically, i.e., occur in individuals without a family history of CRC or inherited genetic mutations that increase the risk of the disease. In general, the main risk factor for CRC is age, however, other factors are associated with the onset of the disease. Both environmental and genetic factors are involved in the risk of developing CRC. Behavioral and dietary habits, such as consumption of red or processed meat, a sedentary lifestyle that leads to overweight and obesity, alcohol consumption, and smoking are the main risk factors. On the other hand, calcium

supplements, consumption of whole grains, fiber, and dairy products, as well as physical activity, are considered protective, particularly for CRC. Patients with a family history of CRC have a higher risk of developing the disease and it corresponds to around 25% of hereditary cases. Hereditary cancer syndromes, such as Lynch syndrome and familial adenomatous polyposis (FAP), corresponded to only 5% of total cases [43,44]. Also, patients with a personal history of inflammatory bowel disease (IBD) or Crohn's disease have, respectively, 3–5 and 2–3 fold increased risk of developing CRC [45].

1.1.2.1. Classification and carcinogenesis

CRC develops when epithelial cells acquire a series of genetic and/or epigenetic changes that enable them to be hyperproliferative. Adenoma–carcinoma sequence is the term used to describe the CRC carcinogenesis that is responsible for the vast majority of sporadic CRC cases. This sequence consists of four major stages: initiation, promotion, progression and metastasis. Initiation involves irreversible genetic damage in normal intestinal epithelial cells, leading to subsequent neoplastic transformation and the formation of a small adenoma. In the promotion stage, the initiated cells proliferate, inducing abnormal growth (neoplasm) and adenoma growth. Subsequently, in the progression phase, by undergoing further genetic and epigenetic alterations, benign tumor cells transform into malignant tumor cells and acquire aggressive characteristics and metastatic potential [44,46]. The metastasis phase is marked by the spreading of cancer cells from the primary organ to other organs or tissues through the bloodstream or the lymphatic system. The duration of each phase is difficult to estimate accurately and has wide ranges. These processes generally take a long time, and decades are required for all stages to be completed in CRC (**Fig 5**) [44].

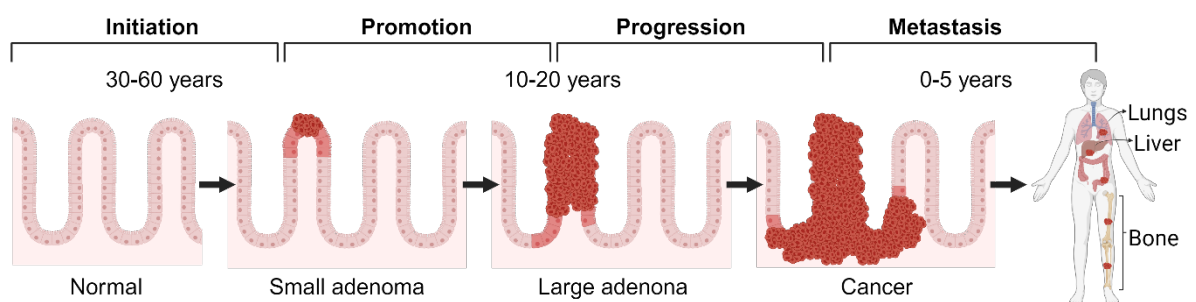


Figure 5: Adenoma–carcinoma sequence described for CRC carcinogenesis. Colorectal carcinogenesis occurs through four stages: initiation, promotion, progression and metastasis. The liver is the most common metastatic site, followed by the lungs and bones. Although the exact duration of each stage is challenging to determine, the entire process typically spans several decades. Adapted from [44]. Image created with BioRender.com.

At least three major molecular pathways can lead to CRC. The most common, responsible for 70-75% of total cases, is the chromosomal instability (CIN) pathway. This pathway is characterized by aneuploidy or structural chromosomal abnormalities, loss of heterozygosity at tumor suppressor gene loci, and chromosomal rearrangements. In these tumors, mutations that inactivate the APC gene are often the early event, leading to the loss of APC function and the constitutive activation of the Wnt signaling pathway. Subsequently, activating mutations in the KRAS gene may occur, affecting patients' response to therapies that target KRAS-dependent signaling pathways. In 20-30% of CRC cases, the cancer develops through the serrated pathway, which involves early mutations in the KRAS and/or BRAF genes. Within this pathway, tumors can develop a microsatellite stable (MSS) phenotype and acquire mutations in the P53 gene, which plays a role in controlling DNA repair and cellular responses to oxidative stress. These tumors can also display a CpG island methylation (CIMP) phenotype, an epigenetic modification occurring at the promoter regions of tumor suppressor genes, such as the DNA mismatch repair protein MLH1, leading to gene silencing. CIMP-positive tumors can be further divided into two types: CIMP-high tumors associated with BRAF mutations and MLH1 methylation, and CIMP-low tumors associated with KRAS mutations. Alternatively, nearly 3-5% of tumors can develop through the microsatellite instability (MSI) pathway due to the inactivation of DNA mismatch-repair (MMR) genes. These tumors are characterized by mutations and loss of function in MMR genes (such as *MLH1*), leading to the accumulation of errors in microsatellite regions. This causes instability in the microsatellite regions and promotes a hypermutable phenotype in tumor cells, that keep copying these errors and acquiring new mutations. These tumors can also display a CIMP phenotype. Since these pathways are not mutually exclusive, tumors can exhibit characteristics from multiple pathways [47].

CRC is a particularly heterogeneous disease, with each tumor exhibiting unique features. Histopathologic variables and molecular pathways alone do not always effectively stratify patients or predict their prognosis and response to treatment. Previously described molecular subtyping methods focus on specific features for certain patient groups. Still, differences in methodology and criteria can result in overlapping and unclear classifications of tumor types and their clinical significance [48]. To overcome this challenge, many research groups have extensively characterized the gene signatures of CRC patients to categorize them into clinically relevant subgroups. Thus, in 2015, the International Colorectal Cancer Subtyping Consortium united efforts to analyze large-scale transcriptomic data and subtyping algorithms, proposing a gene expression-based classification system that distinguishes CRC into four consensus molecular subtypes (CMS): CMS1, CMS2, CMS3, and CMS4 (**Fig 6**) [49].

CMS1 corresponds to 14% of total CRC tumors. These tumors have hypermutable characteristics and are frequently associated with MSI and CIMP-high phenotype with BRAF often mutated, low somatic copy number alteration (SCNA), immune infiltration, and worse prognosis after relapse. The CMS2 tumors follow the “canonical” CRC carcinogenesis pathway and have high SCNA, MSS, activated Wnt & MYC pathway, and mutated TP53 gene. These correspond to 37% of all CRC cases. The CMS3, on the other hand, corresponds to 13% of CRC cases and it is called the metabolic subtype due to the deregulation of metabolic pathways. These tumors have low to moderate MSI, intermediate CIMP status and KRAS mutation. The CMS4 or the mesenchymal subtype tumors have upregulation of genes involved in epithelial-to-mesenchymal transition (EMT), matrix remodeling, angiogenesis, TGF β signaling, and inflammatory-related systems. This subtype corresponds to 23% of CRC cases. The remaining 13% of total cases are considered to have mixed features and could represent a transition phenotype, heterogeneity among tumors, hypothetical mixtures between subtypes or an indeterminate CMS subtype. However, these cases are not considered a fifth independent subtype [49-51].

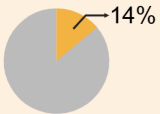
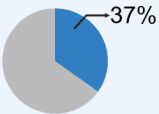
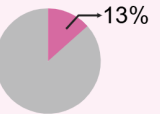
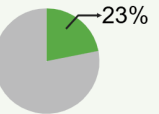
CMS1	CMS2	CMS3	CMS4
MSI Immune	Canonical	Metabolic	Mesenchymal
			
 Proximal Colon	 Distal Colon to rectum	 No specific localization	 Distal Colon to rectum
Serrated adenoma	Tubular adenoma	Tubular adenoma	Serrated adenoma
MSI CIN ⁻ CIMP ^{high} BRAF mutations	MSS CIN ⁺ CIMP ⁻	MSS CIN ⁻ CIMP ^{low} KRAS mutations	MSS CIN ⁺ CIMP ⁻
Immune infiltration and DNA damage repair	Epithelial differentiation Wnt and MYC activation	Metabolic desregulation	TGF- β and EMT activation Stromal infiltration Angiogenesis
Good prognosis; Poor prognosis after recurrence			Dismal Prognosis

Figure 6: Consensus Molecular Subtypes (CMS) classification by the International Colorectal Cancer Subtyping Consortium. This Classification reflects the biological differences in the gene expression-based molecular subtypes. Adapted from [49,50]. Image created with BioRender.com.

MSI – microsatellite instability; MSS – microsatellite stable; CIMP – CpG island methylator phenotype;

1.1.2.2. Diagnosis and treatment

Early diagnosis is a crucial factor to improve the survival of CRC patients, however the diagnosis at an early stage is not easy as initial stages are often asymptomatic [52]. Regular screening of the population can reduce CRC incidence and mortality, as early detection enables the removal of pre-cancerous lesions before malignant transformation or enables a more effective treatment response. Several screening modalities are available and widely adopted globally. For instance, fecal occult blood test, which checks stool samples for small amounts of blood, can indicate the presence of pre-cancerous lesions or cancer [43]. Similar to GC, there are no specific biomarkers that can diagnose CRC. They are usually proteins associated with malignancy and might be clinically useful in patients with cancer. The most classical markers accepted in routine clinical practice are the CEA, CA19-9, the tissue polypeptide specific antigen (TPS), CA72-4, and hematopoietic growth factors (HGF-s). Nevertheless, most of these are not sensitive enough and are mostly used as prognostic markers [52,53]. Therefore, these current non-invasive screening tools are, in most cases, not sensitive enough to detect pre-cancerous lesions and may miss significant early CRC. Thus, the main screening method for CRC is the very invasive method of colonoscopy, a highly sensitive test that involves high costs, resources and specialized medical doctors [44,53]. Detection of KRAS mutations is also largely used and is extremely important to determine the correct treatment and targeted therapy administered to CRC patients [53].

After a diagnosis of CRC, treatment involves a multimodal approach tailored to specific characteristics of the tumor. Factors such as the tumor's location, size, extent of metastasis, and the patient's overall health condition are all considered in determining the appropriate treatment plan [45]. At early-stage CRC, i.e., patients with no metastatic disease, laparoscopic surgery for primary CRC is the most common approach. In fact, for these patients, chemotherapy has not been found to provide a great advantage in their overall survival [45,54]. In patients with potentially resectable metastatic disease, chemotherapy is administered to reduce the number and size of the metastases and allow a subsequent surgical resection. When patients present disseminated unresectable disease, the treatment becomes palliative rather than curative, with the main intention of reducing the symptoms, aggressiveness, and extension of the disease. The first-line chemotherapy includes fluoropyrimidines (e.g., 5-FU or capecitabine) alone or combined with platinum such as oxaliplatin. Leucovorin could also be administered, helping to reduce the toxicity of the treatment and enhancing the effectiveness of 5-FU [45,55,56]. Radiation therapy is generally not recommended as a routine treatment for CRC patients, being primarily used in RC. It is mainly applied to patients with advanced diseases where tumor

resection by surgery is not possible or is incomplete. In these cases, radiation therapy is often combined with the administration of fluoropyrimidines [57].

In more advanced stages of the disease, a monoclonal antibody Bevacizumab that targets the vascular endothelial growth factor (VEGF) is used in combination with traditional chemotherapy and has been shown to improve patient outcomes. Bevacizumab selectively binds circulating VEGF, preventing its binding to the VEGF receptor. This disrupts the signal transduction pathway responsible for angiogenesis, proliferation, and migration of cancer cells [45,58]. On the other hand, Cetuximab and Panitumumab are other two approved monoclonal antibodies that target epidermal growth factor receptor (EGFR). This receptor is involved in signaling pathways critical to cancer cell proliferation and survival. However, in contrast to Bevacizumab, which is used in all patients, Cetuximab and Panitumumab are only effective in tumors without KRAS mutations [58], since these lead to the constitutive activation of signaling pathways independent of EGFR activation. Additionally, some patients eligible for this treatment do not respond to anti-EGFR monoclonal antibodies or acquire resistance during treatment [58,59].

2. Glycosylation in health and cancer

Glycosylation is the enzymatic process responsible for the assembly of glycans (also known as carbohydrates or saccharides) that can exist as free molecules or attached to proteins, lipids and RNA (although still poorly studied). Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus within the cells, and it is mediated by the organized action of a collection of different glycosyltransferases (GTs) and glycosidase enzymes forming a diversity of glycoconjugates [60,61]. The generated glycoconjugates construct the glycosylation repertoire, or glycome, of a cell that tightly regulates a vast of vital biological functions. Thus, the glycosylation process is crucial for recognition, signaling, interaction inside the cell and/or between cells, cell-matrix interaction and adhesion, protection against protease activity, and immune recognition, among countless others [60,62].

Apart from the hundreds of enzymes involved in the biosynthesis of the diverse glycan structures, the glycosylation process is closely dependent on the ability of the cell to create the GTs substrates: the sugar donors. These are the activated forms of monosaccharides, which are the fundamental units in glycan biosynthesis. The sugar donors can be salvaged from recycled glycoconjugates by lysosomal glycosidases (salvage pathway) or converted enzymatically at the cytoplasm mainly from imported glucose, galactose and fructose residues (*de novo* pathway). The derived monosaccharides are then activated to high-energy sugars through many enzymatic steps, culminating in nucleotide

addition, commonly of uridine diphosphate (UDP), guanosine diphosphate (GDP), or cytidine monophosphate (CMP). These activated sugars are then transported into the Golgi and the ER, where they act as glycan building block units for glycans biosynthesis in cells. Furthermore, in addition to the ER and Golgi, transporters presented on the cell surface and lysosomes ensure the proper trafficking and availability of sugar donors for glycan synthesis, playing an essential role throughout the glycosylation process [63] (**Fig 7**).

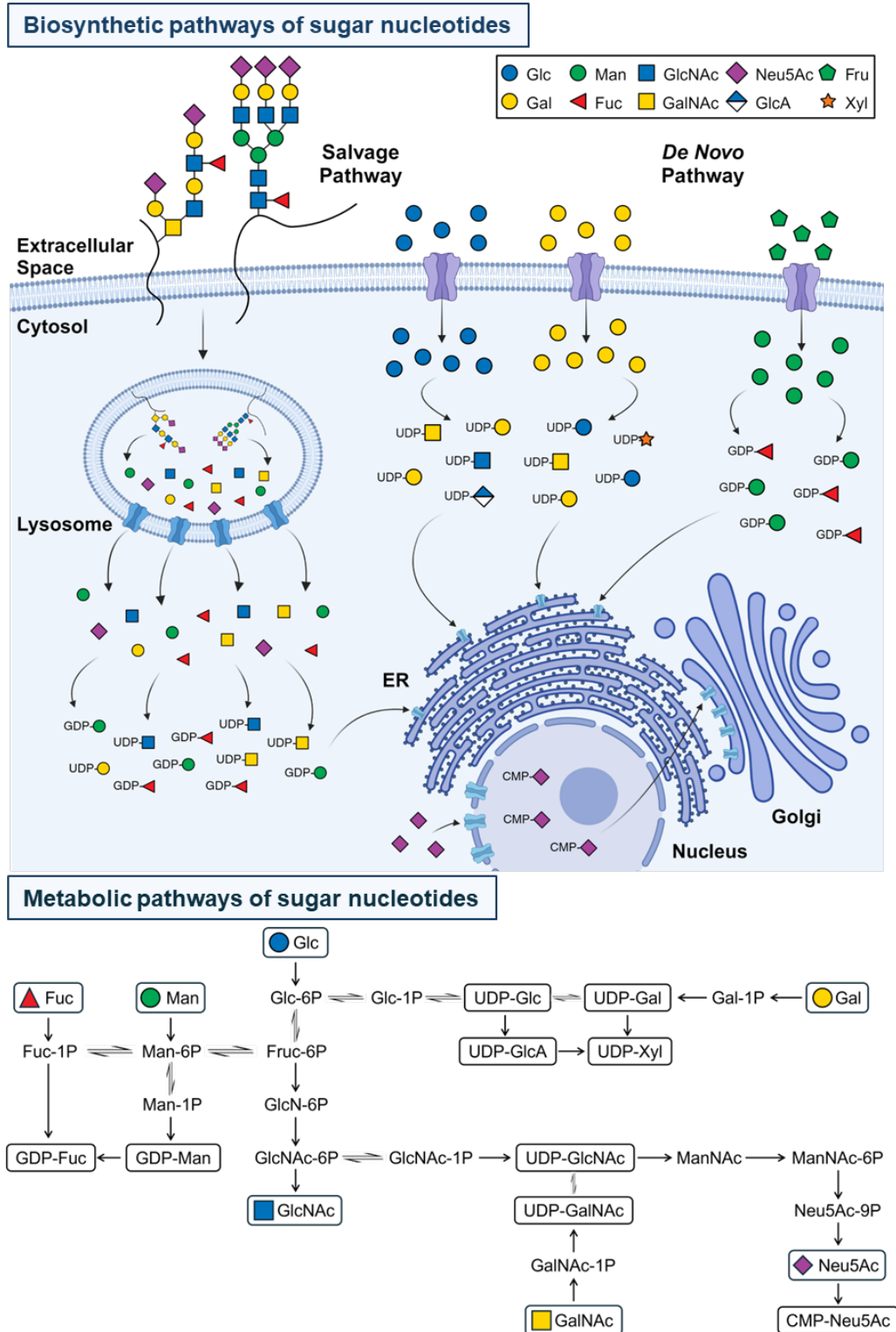


Figure 7: Schematic representation of the biosynthetic and metabolic pathways involved in the synthesis of sugar nucleotide donors. Monosaccharides are derived from glycans released from degraded glycoproteins at lysosomes (salvage pathway) or from free residues available at the cell's cytosol resulting from the enzymatic conversion of imported glucose, galactose and/or fructose (*de novo* pathway). Activated forms of the monosaccharides or sugar donors are acquired by enzymatic addition of nucleotides, commonly UDP, GDP, or CMP, to the sugars. Image created with BioRender.com.

CMP – cytidine monophosphate, Fuc – Fucose, Gal – Galactose, GalNAc – *N*-Acetylgalactosamine, GDP – Guanosine diphosphate, Glc – Glucose, GlcA – Glucuronic Acid, GlcNAc – *N*-Acetylglucosamine, Man – Mannose, Neu5Ac – *N*-acetylneuraminic acid, UDP – Uridine diphosphate, Xyl – Xylose.

2.1. Different types of glycosylation

Glycoconjugates, depending on the nature of their non-glycan counterpart and glycan structure composition, can be divided into glycoproteins, proteoglycans, glycosphingolipids, and the recently identified glycoRNA (Fig 8).

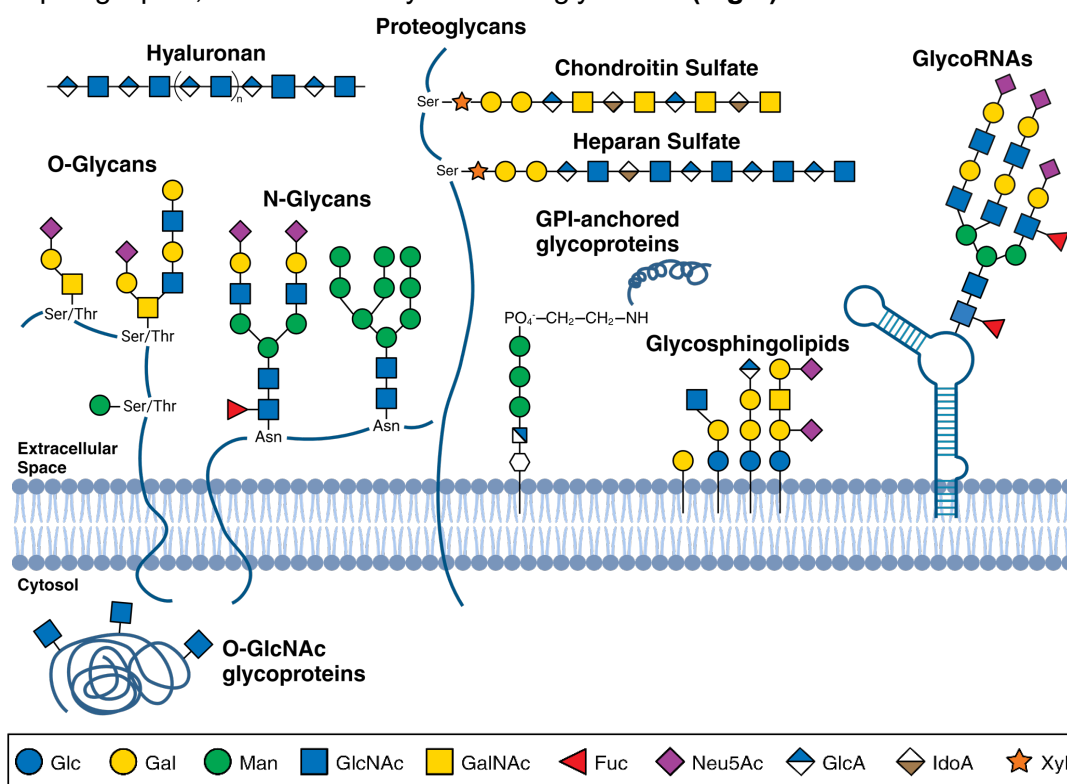


Figure 8: Schematic representation of the different glycoconjugates found in human cells. According to the molecule to which glycans are attached and the type of glycan structure formed, several classes of glycoconjugates are defined including glycoproteins, in which glycans are attached to asparagine (*N*-glycans) or serine/threonine (*O*-glycans) amino acids; proteoglycans, where long glycosaminoglycan (GAG) chains are attached to proteins; glycosylphosphatidylinositol (GPI)-anchored glycoproteins, in which a protein is attached to a GPI structure that, in turn, is attached to membrane lipids; glycosphingolipids, whose glycans are attached to lipids; and glycoRNAs, recently reported and poorly understood consisting of glycans attached to cell surface small non-coding RNAs. Created with BioRender.com.

Fuc – Fucose, Gal – Galactose, GalNAc – *N*-Acetylgalactosamine, Glc – Glucose, GlcA – Glucuronic Acid, GlcNAc – *N*-Acetylglucosamine, IdoA – Iduronic Acid, Man – Mannose, Neu5Ac – *N*-acetylneuraminic acid, Xyl – Xylose.

Protein glycosylation represents the most abundant type of post-translational modification (PTM), being estimated that over 50% of all eukaryotic proteins undergo glycosylation [64]. In glycoproteins, specific amino acid residues of the protein backbone are modified with carbohydrate chains through both *N*- and *O*- types of linkages. In *N*-glycosylation, the attachment of oligosaccharide chains to the polypeptide backbone is mediated through the nitrogen (N) atom of the asparagine (Asn) residue occurring in Asn-X-Ser/Thr consensus sequence, in which X can be every amino acid except proline. This type of glycosylation initiates in the cell's ER, where an oligosaccharide composed of 14 sugars (Dolichol-Linked Mannose Precursor) is transferred *en bloc* and is covalently attached to an Asn residue within the canonical consensus sequence, by an oligosaccharyltransferase (OGT). The next phase starts in the ER and continues into the Golgi apparatus, where a succession of trimming, elongation, and capping modifications leads to a variety of different glycoforms (Reviewed in [65,66])

On the other hand, the attachment of carbohydrate structures to the peptide chain of *O*-linked glycoproteins, via the oxygen (O) atom of both serine (Ser) or threonine (Thr) amino acid residues is called *O*-glycosylation. The most common type is *O*-GalNAcylation, also known as mucin-type *O*-glycosylation due to its vast presence at the cell surface and secreted mucins. This process happens in the Golgi apparatus and is initiated with the covalent attachment of a *N*-acetylgalactosamine (GalNAc) monosaccharide to the Ser or Thr residue forming the Tn antigen. Contrarily to *N*-glycosylation, there is no known consensus sequence for this modification, which can be accomplished by one of twenty distinct polypeptide GalNAc transferases (ppGalNAcTs). The Tn antigen is further extended into one of multiple *O*-glycan core structures (1-8). The core 1 or the T antigen, is formed following the addition of a galactose (Gal) monosaccharide by the C1GalT1 glycosyltransferase. Core 1 structures can be further sialylated, giving rise to the sialyl-T or disialyl-T antigens, or modified by core 2 β 1,6-*N*-acetylglucosaminyltransferase (C2GnT), which adds *N*-acetylglucosamine (GlcNAc) to the T antigen, forming core 2 structures. Adding one or two GlcNAc residues to the Tn antigen gives rise to core 3 and core 4 structures, respectively. The addition of *N*-acetylglucosamine (GlcNAc) repeating units to the above-described core structures allows the elongation, or extension, of *O*-glycan chains. These structures can then be further modified by different glycosyltransferases, including sialyltransferases (STs) and fucosyltransferases (FUTs), forming increasingly complex terminal structures (Reviewed in [66,67]).

While both *N*-glycosylation and *O*-GalNAcylation happen in the ER and Golgi, *O*-GlcNAcylation (GlcNAc-type *O*-glycosylation) takes place in the cytoplasm, nucleus and mitochondria. In this type of glycosylation, proteins residing within the cytosolic, mitochondrial, and nuclear cellular compartments have a single *O*-linked GlcNAc

monosaccharide at a specific peptidic glycosylation site (glycosites). The O-GlcNAc is dynamically added and removed from proteins by just two enzymes, the O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA), respectively. This type of glycosylation is a nutrient and stress responsive PTM and can regulate important cellular processes such as gene expression, signal transduction, cell cycle, protein homeostasis, cellular stress response, and neuronal function (Reviewed in [68]).

Proteoglycans are another type of protein glycoconjugate. It consists of a “core” protein and one or more covalently attached glycosaminoglycan (GAGs) chains. GAGs are linear polysaccharides composed of repeating disaccharide units, each consisting of an amino sugar (GlcNAc, or GalNAc) and a uronic acid (glucuronic acid [GlcA] or iduronic acid [IdoA]) or a Gal. According to the disaccharide units that build these chains, GAGs can be classified as Heparan Sulfate (HS), Chondroitin Sulfate (CS), Dermatan Sulfate (DS) or Keratan Sulfate (KS) (Reviewed in [68-70]). Hyaluronan (HA) is the only non-sulfated GAG that is an exception because it lacks a covalent bond to a protein core and is secreted “free” to the extracellular matrix [71].

Cell membrane lipids (like phosphatidylinositol) can also have glycans attached, in a common glycosylphosphatidylinositol (GPI) structure, which in turn is attached to proteins, forming GPI-anchored proteins [72]. Additionally, glycans can be linked to other lipidic structures, such as ceramide molecules, originating glycosphingolipids (GSLs). The biosynthesis of GSLs occurs through the stepwise addition of sugars to ceramide, producing either galactosylceramide (GalCer) or glucosylceramide (GlcCer), depending on whether it is a β -linked galactose or glucose. The elongation process results in a variety of combinations that subclassify GSLs into subgroups: neutral (containing no charged sugars or ionic groups), sulfate, or sialylated GSLs (having one or more sialic acid residues). The latter group is generally known as gangliosides (GDs) and often shares terminal structures with glycoproteins such as Lewis structures (reviewed in [73]).

Finally, very recently, glycoRNAs were also reported to be present at the cell membrane *in vitro* and *in vivo*. It consists of highly sialylated and fucosylated *N*-glycans attached to small noncoding RNAs [74] in the modified RNA base 3-(3-amino-3-carboxypropyl)uridine (acp3U) [75]. A recent study suggests that cell surface glycoRNAs play a critical role in neutrophil recruitment *in vivo* [76]. Nonetheless, it is important to mention that there is still a debate within the scientific community about this type of glycosylation.

2.2. Glycosylation in cancer

The glycosylation pattern in healthy cells is a highly controlled process, presenting tissue specific patterns allowing their normal function. Thus, it is not surprising that changes

in glycosylation are often seen in both pre-cancerous and cancerous conditions. In fact, this change can actively contribute to the transformation of normal cells and tissues into cancerous ones.

In cancer cells, the abnormal glycosylation can result from various disrupted molecular mechanisms, such as changes in the expression, activity, and location of certain GTs and molecular chaperones within the cell, as well as imbalances in the availability of donor substrates. In the literature, it is well-documented the expression of several glycan alterations in cancer. Increased *N*-glycan branching, due to upregulation of the *N*-acetylglucosaminyltransferase V (MGAT5) enzyme, changes in sulfation patterns and overexpression of GAGs, expression of truncated *O*-glycans (such as Tn and Sialyl Tn antigen), and increased sialylation and/or fucosylation, frequently associated with the appearance of sialyl Lewis's antigens in glycolipids and glycoproteins, are the most typical modifications (**Fig 9**) (reviewed in [60,77-80]). These alterations are reported to be important across the several steps of cancer development, progression, and other key biological processes, such as cell migration, invasion, metastization process, tumor angiogenesis, host antitumor immunity and cancer cell survival. Consequently, the vast majority of cancer-associated glycans are connected with more aggressive tumors, and therefore with worse patient prognosis and poor clinical outcomes [60,81]. Hence, understanding the mechanisms behind changes in glycosylation is crucial to clarify the role of altered glycans in cancer development. This knowledge can also help identifying potential molecular targets for developing more effective therapies and new biomarkers.

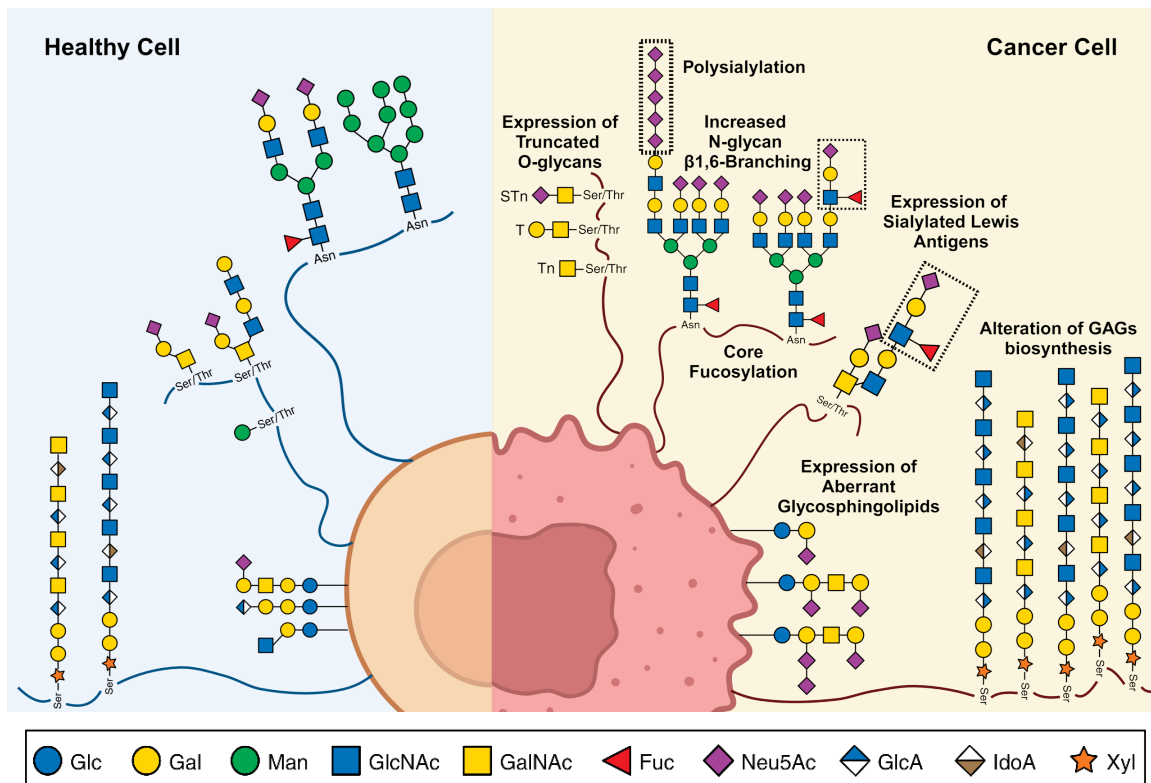


Figure 9: Glycoconjugates found at the surface of normal versus cancer cells in humans. Altered glycosylation is a feature in cancer, and the most reported cancer-associated glycans are the expression of truncated O-glycan structures, the aberrant sialylation of glycosphingolipids, as well as increased sialylation of glycoproteins (through both polysialylation and expression of sialylated Lewis glycans), core fucosylation, overexpression and altered sulfation of GAGs, and β 1,6-branching of *N*-glycans. Created with BioRender.com. Fuc – Fucose, Gal – Galactose, GalNAc – N-Acetylgalactosamine, Glc – Glucose, GlcA – Glucuronic Acid, GlcNAc – N-Acetylglucosamine, IdoA – Iduronic Acid, Man – Mannose, Neu5Ac – N-acetylneuraminic acid, Xyl – Xylose.

2.2.1. The Lewis antigens and the role of SLeX in cancer

The Lewis antigens comprise a family of fucosylated structures commonly found at the terminal positions of several glycoconjugates, such glycoproteins and glycolipids, and have an important role in many cellular functions in health and cancer [82]. These antigens are characterized by a LacNAc core structure composed of a GlcNAc residue linked to a Gal residue. Based on the nature of this linkage, Lewis antigens are classified into two types: Type I (Gal β 1,3GlcNAc) and Type II (Gal β 1,4GlcNAc) structures. Then, FUTs and STs can act sequentially during the biosynthesis of these antigens, originating a myriad of structures (**Fig 10**) [60,82,83]. As previously mentioned, altered expression of GTs is one of the main causes of aberrant glycosylation in cancer [60]. Especially an upregulation of STs is well documented in cancer and leads to the overexpression of sialylated structures, such as the sialylated Lewis antigens. STs are responsible for the terminal enzymatic addition of a sialic acid (glycan capping) to other carbohydrates by glycosidic bonds, being *N*-acetylneuraminic acid (Neu5Ac) the most common sialic acid found in mammals. This enzymatic process is carried out by more than 20 different Golgi-resident STs that can perform sialic acid additions to carbon atoms at position C3 (ST3Gal-VI), C6 (ST6Gal, II and ST6GalNAc-VI), or C8 (ST8Sial-VI) of carbohydrates, in α 2-3, α 2-6, or α 2-8 linkages, respectively [84]. Regarding sialylated Lewis antigens, the overexpression of ST3Gal enzymes results in the biosynthesis of Sialyl Lewis X (SLeX) and SLeA glycan structures, commonly found in cancer.

Focusing on SLeX, this structure is a terminal tetrasaccharide commonly found at the cell surface attached to both *N*- and *O*-linked glycans on proteins and glycolipids. SLeX biosynthesis requires the sequential addition of an α 2-3 linked sialic acid (Neu5Ac) by a member of the ST3Gal family (mainly the ST3Gal III, IV and VI) to a Gal residue on a type II glycan chain, followed by the addition of an α 1-3 linked fucose to the GlcNAc residue by a fucosyltransferase, mainly the FUTIII/V/VI/VII [83,85] (**Fig 10**).

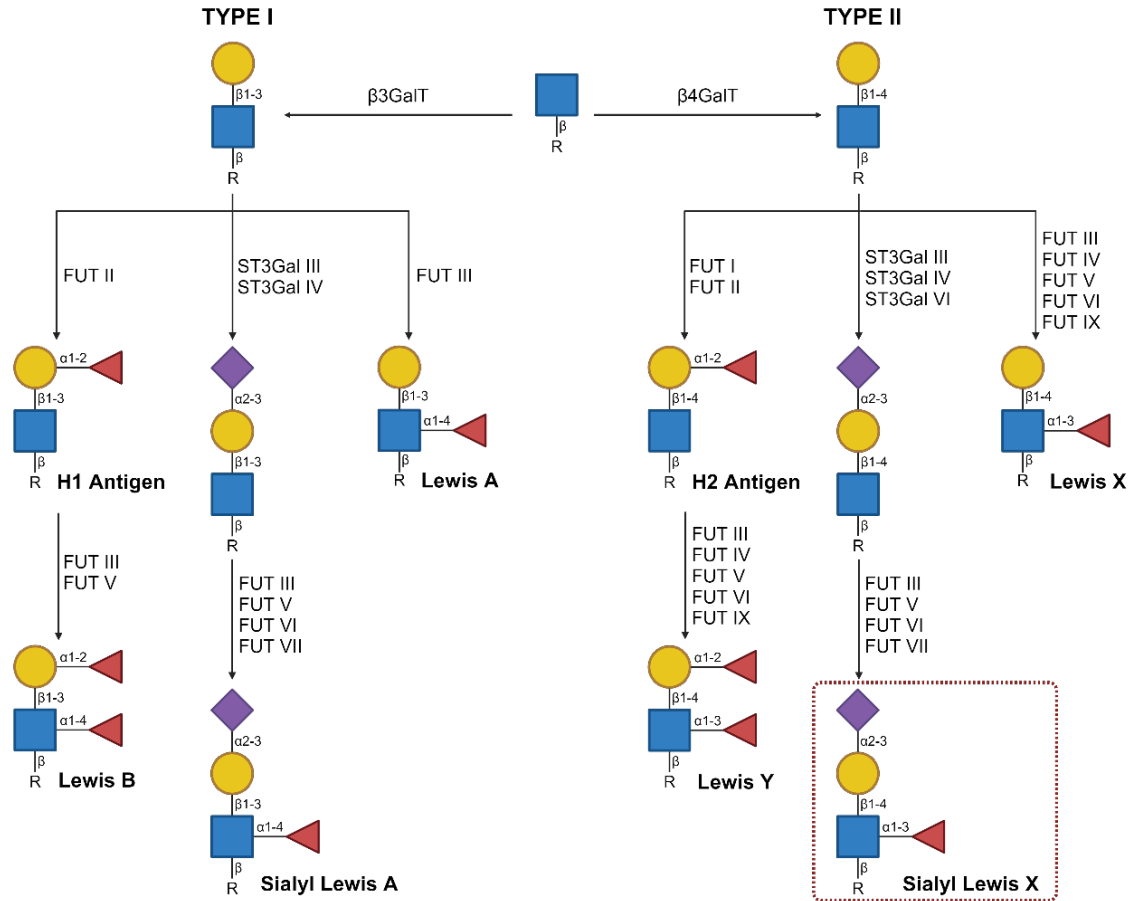


Figure 10: Schematic representation of the biosynthetic pathways of the several structures belonging to the Lewis antigens group, with a highlight for the SLeX biosynthesis. Image created with BioRender.com. Gal – Galactose; GlcNAc – N-Acetylglucosamine; Fuc – Fucose; Neu5Ac – N-acetylneuraminic acid; β 3GalT – β 1,3-galactosyltransferase; β 4GalT – β 1,4-galactosyltransferase; FUT – fucosyltransferase; ST3Gal – α 2,3-sialyltransferase; R – variable carbohydrate.

SLeX is a key glycan structure involved in the recognition of cell adhesion molecules, particularly the selectin family [86]. In fact, SLeX interaction with selectins is crucial in leukocyte inflammatory response [87]. Selectins are cell adhesion receptors expressed on leukocytes, endothelial cells and platelets, and are classified based on their expression sites - L, E, and P - respectively. These C-type lectins can bind to glycoconjugates that carry terminal SLeX and SLeA structures, playing a central role in initiating the tethering of leukocytes during inflammation. The initial inflammatory response is characterized by the release of different interleukins that induce the expression of selectins on endothelial cells and SLeX expression at the cell surface of leukocytes. SLeX on leukocytes binds to E-selectin, promoting leukocyte attachment to the vascular endothelium. This interaction initiates leukocyte rolling along the vascular epithelium, allowing immune cells to migrate through the endothelial barrier and extravasate into tissues where they are needed [87-91]. Research shows that tumor cells exploit similar mechanisms to those used by leukocytes

for vascular extravasation and migration to distant organs, facilitating metastasis [91]. For metastasis to occur, the cancer cells need to adhere to the vascular endothelium. This initial adhesion is mediated by the interaction of E-selectins on endothelial cells with aberrantly expressed SLeX on cancer cells. This binding facilitates cancer cell adhesion, tethering, and subsequent extravasation [87,88,91,92]. Additionally, SLeX plays a significant role in cancer cell survival in circulation. When cancer cells enter circulation, they often associate with platelets that express P-selectin. The interaction between P-selectin on platelets and SLeX on cancer cells shield the tumor cells from immune recognition, protecting them from destruction and facilitating their spread to distant organs [90,93,94] (**Fig 11**). SLeX also influences angiogenesis [92,95,96]. In fact, inhibition of SLeX, using antibodies, has been shown to reduce the formation of tube-like networks of endothelial cells, when co-cultured with SLeX-positive cancer cells [95]. In hepatocarcinoma cells, angiogenesis was inhibited by interfering with SLeX biosynthesis [92,96]. These processes collectively enhance cancer metastasis.

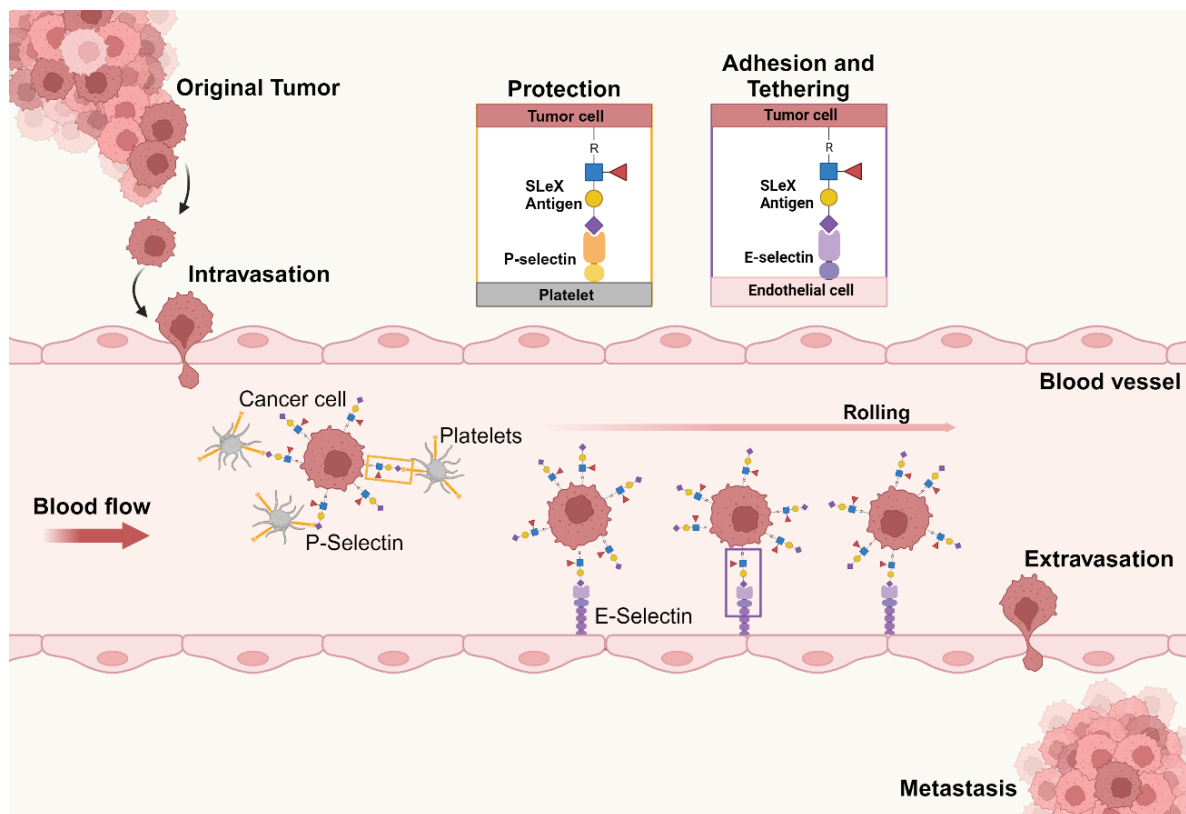


Figure 11: Role of SLeX in cancer cell metastatic spread. SLeX expressed in cancer cells interacts with E-selectin on endothelial cells, leading to the adhering of tumor cells to vascular endothelium. The cells are pulled along with the blood flow, rolling through the vascular epithelium. SLeX also interacts with P-selectin expressed on platelets, protecting the cancer cells from immune system attack, and avoiding apoptosis. Image created with BioRender.com.

Therefore, numerous studies have demonstrated a strong association between SLeX expression and increased metastasis, along with poor prognosis in various cancer types. In prostate cancer, for instance, knockdown of the FUTIII enzyme effectively abolished SLeX expression on the cancer cell surface, reduced E-selectin binding, and diminished cell adhesion and rolling on the blood vessel wall [97]. Similarly, in GC cells, overexpression of ST3GalIV increased SLeX biosynthesis, correlating with a more invasive phenotype both *in vitro* and *in vivo* [98]. Analysis of GC tissue samples further confirmed a positive correlation between α -2,3-sialylation and cancer stage, metastasis, and tumor invasion, reinforcing the role of SLeX in promoting invasive cancer cell behavior [99,100]. In bladder cancer, SLeX expression is closely linked with the invasive and metastatic potential of the cancer cells [101]. A recent study found elevated levels of FUTVII and SLeX that promote the growth and invasion of bladder cancer cells. SLeX was enriched in small extracellular vesicles, and this could disrupt the tight junctions and lead to increased vascular permeability in Human Umbilical Vein Endothelial cells [102]. In lung metastasis models, silencing of ST3GalIV inhibited SLeX expression, reducing cell adhesion to endothelial cells [103]. A similar pattern was observed in CRC, where EMT, a critical step in metastasis, was correlated with the upregulation of enzymes like FUTIII, ST3GalIII, and ST3GalIV, which are involved in the biosynthesis of SLeX and SLeA [104]. In pancreatic ductal adenocarcinoma, ST3GalIII and ST3GalIV are also overexpressed, and their knockdown significantly reduced SLeX expression, impairing cell migration, invasion, and binding to E-selectin [105].

Recently, using an *in vitro* ST3GalIV overexpression GC cell model, our group identified CEA as the major protein carrier of SLeX [100] and validated its expression in GC tissues correlating with metastatic disease and poor prognosis of patients. This finding aligns with previous studies demonstrating the significant role of CEA in cancer metastasis. For instance, in CRC, CEA has been reported to act as a mediator for rolling on E- and L-selectin [106]. CEA can also bind and activate endothelial cells triggering signaling pathways involved in adhesion, invasion, and tumor angiogenesis [107,108]. Additionally, several studies have revealed that CEA promotes cancer cell metastasis through interaction with the hnRNPM4 receptor in liver Kupffer cells [109,110]. This interaction triggers cytokine release and the tyrosine phosphorylation of proteins, creating an inflammatory environment that facilitates metastasis formation in the liver [111,112]. These findings emphasize the importance of identifying protein carriers, like CEA, which could serve as biomarkers for cancer metastasis.

2.2.2. Role of truncated O-Glycans in cancer

In cancer, the *de novo* expression of truncated O-glycans, such as Tn and STn, is commonly detected. In fact, STn is used as a clinical biomarker, as mentioned previously, in GIC tumors as a prognostic marker. This antigen is rarely expressed and detectable in normal tissues. However high levels of STn have been widely reported across different epithelial-derived tumors, such as GIC [113-116], pancreatic [106,117], breast [118,119], and bladder [120], among others [121]. The synthesis of these glycans results from impaired O-glycan extension. It is accepted that overexpression of the enzyme ST6GalNAcI, which adds a sialic acid residue to Tn antigen, and/ or the mutation of the C1GalT1-specific chaperone 1 (C1GALT1C1 or COSMC) leads to impaired O-glycosylation extension (**Fig 12**). The COSMC chaperone has an essential role in the stabilization of the C1GalT1 at the Golgi, necessary for core 1 formation [118,122]. Additionally, the initiating enzymes ppGalNAcTs have an altered expression in cancer, which by itself already affects the normal density and pattern of the O-glycan in proteins. Furthermore, Bard and colleagues describe that translocation of ppGalNAcTs to the ER can increase O-glycans site occupancy, leading to impaired elongation steps and accumulation of Tn and STn antigens [123].

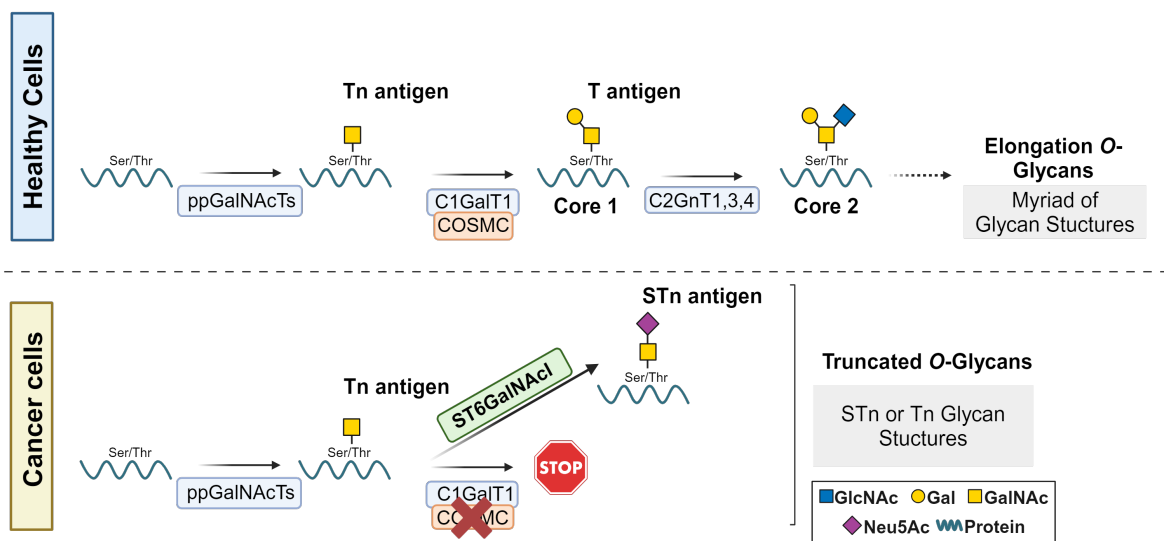


Figure 12: Biosynthesis of truncated O-glycans in cancer cells. In healthy cells, the ppGalNAcT enzyme family initiates O-GalNAcylation by adding GalNAc to serine or threonine residues, which results in the formation of the Tn antigen. Subsequently, the C1GALT1 enzyme adds a Gal residue forming the T antigen (core 1). The C2GnT enzyme may then extend this structure by adding GlcNAc, leading to core 2, which can be further elongated into a variety of complex glycan structures. In cancer cells, mutations or loss of the COSMC gene (C1GALT1C1, necessary to the normal function of C1GalT1) and/or overexpression of ST6GalNAcI halts O-glycan elongation, resulting in the overexpression of truncated glycans such as the Tn and STn antigens. Image created with BioRender.com.

GlcNAc – *N*-Acetylglucosamine; Gal – Galactose; GalNAc – *N*-Acetylgalactosamine; Neu5Ac – *N*-acetylneuraminic acid;

In general, the presence of these antigens, especially STn is correlated to more aggressive tumors and a poorer prognosis of cancer patients. In several types of cancer, STn was associated with decreased cancer cell adhesion, increased tumor growth, increased tumor cell migration, invasion, and poor prognosis [120,121,124]. Focusing on GIC, STn-expressing cells have a more aggressive cell behavior, due to changes in the EMT process, degrading and invading the surrounding extracellular matrix (ECM) and reducing cell-cell adhesion capacity through the loss of E-cadherin expression and to chemotherapy resistance [113,117,121].

Furthermore, STn can be carried by various glycoproteins in different cellular compartments, its role in cancer progression may vary across different tumor types [121]. Proteins such as MUC1 [125-127], CD44 [127-129], integrin $\beta 1$ [130], and osteopontin [131] have been identified as STn carriers, and all of these proteins play roles in cell adhesion, migration, or chemotaxis. Thus, it is believed that the expression of STn in these glycoproteins may influence their function and contribute to cancer progression. Thus, targeting STn and its carrier proteins is a promising approach for cancer therapy [121].

3. Strategies for targeting cancer-associated glycans as promising future therapies

Currently, cancer treatment options include surgery, when resection is possible, along with chemotherapy and/or radiotherapy. When eligible and available, targeted therapies directed to cancer cells' specific molecular markers, such as monoclonal antibodies (mAbs) and small molecule inhibitors, have become integral components of clinical practice. Small molecules, primarily receptor kinase inhibitors, and mAbs are approved by both the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA), with numerous clinical trials underway for new therapies. While these treatments have proven beneficial, they face several challenges, such as being restricted to a small subset of cancer patients, associated toxic side effects, and the emergence of therapy resistance [132-134]. Thus, the need for alternative novel and unexplored molecular targets, such as cancer-associated glycans, holds significant potential for cancer therapy, offering new avenues for new treatment development.

In fact, glycosylation also plays a significant role in therapy resistance. For example, resistance to the chemotherapeutic agent doxorubicin (DXR), a front-line treatment for certain cancers, is attributed to glycosylation at cell surface proteins, which can affect epitope accessibility and hinder drug binding to target receptor proteins. Additionally, cancer-associated glycans on the surface of cancer cells can structurally obstruct the accessibility of therapeutic antibodies to their protein targets. A notable example is the

HER2 antibody, where it has been demonstrated that deglycosylation of membrane proteins enhances antibody accessibility to the HER2 receptor. Our group recently showed that aberrant α 2,6-sialylation, mediated by ST6Gall, contributes to the resistance to the monoclonal antibody targeting HER2, trastuzumab, in ErbB2-driven GC cells [135]. Similarly, it was demonstrated that *N*-glycans hinder the recognition of PD-L1 by antibodies on the surface of tumor cells, preventing the antibodies from binding to specific glycoforms of PD-L1 [136].

The generation of vaccines and antibodies directed to cancer-associated glycans and glycoproteins have also largely been studied, representing some of the first attempts to target glycans in cancer therapy [126,137,138]. This field of research has provided the basis for recent advanced pre-clinical and clinical studies, being therapeutic options in several types of cancers [139-142]. More recently, several studies using genetically modified T cells expressing chimeric antigen receptors (CAR-T) have also used glycans as targets. For instance, Posey and colleagues successfully induced cancer cell cytotoxicity in xenograft models of T-cell leukemia and pancreatic cancer using a specific CAR that recognized the cancer-associated Tn glycoform of MUC1 (NCT03633773) [143]. Additionally, several phase I/II clinical trials are evaluating the therapeutic potential of GD2-targeting late-generation CAR-T cells in the treatment of various GD2-expressing solid tumors [144,145] (NCT03356782; NCT03635632; NCT04539366). Currently, CAR-Ts targeting Le^y are under evaluation for the treatment of solid and liquid tumors positive for this glycan [146,147] (e.g., NCT03198052, NCT03851146). Despite these advances, the major challenge in the CAR-T field remains the difficulty of effectively targeting solid tumors as well as overcoming toxic effects [147].

The development of Antibody-Drug Conjugates (ADCs) to deliver toxic drugs (payload) to specific tumor cells that exhibit specific antigens (target) has also been explored. Thirteen ADCs were already approved for cancer therapy, and several are undergoing clinical trials (reviewed in [148]). Researchers have explored the abnormal glycosylation patterns in cancer cells as a target for drug delivery. ADCs designed to recognize these glycans have shown promising results both *in vitro* experiments and in animal studies [149-151]. Another strategy, developed by Xiao and colleagues, combines the targeting of HER2-positive cells using trastuzumab, with a sialidase as the payload. This antibody-enzyme conjugate selectively changed the tumor cell glycocalyx, precisely the sialome, resulting in increased activation of natural killer cells with consequently increased cytotoxicity against tumor cells [152].

Besides the above-mentioned strategies, the search for glycosylation inhibitors is emerging as a novel approach in the development of new cancer therapies. High-throughput screening (HTS) of large compound libraries, including natural and chemical

compounds, holds significant potential and is gaining momentum in the glycobiology field. This approach allows the identification of molecules that specifically target glycosylation processes, offering a new class of therapeutic agents [132]. Moreover, recent studies have explored the use of modified sugars, particularly fluorinated analogs, that disrupt glycosylation pathways. These modified sugars have shown promising results in preclinical models as novel cancer therapies by interfering with the abnormal glycan synthesis observed in cancer cells. These innovative strategies open new avenues for targeting aberrant glycosylation involved in cancer progression and metastasis, expanding the arsenal of cancer treatment options.

3.1. High Throughput Screening targeting glycosylation

HTS has revolutionized the drug screening process, enabling the analysis of thousands of compounds efficiently at a lower cost [132]. In fact, HTS approaches have had a notable impact on scientific research and biological testing, with reasonable success rates in obtaining a hit, confirmed by more than one assay *in vitro* and *in vivo* [153,154]. However, only a few drugs from HTS make their way to clinical trials and, when they do, often fail. Common miscalculations in these strategies include 1) poorly validated targets, 2) small screening libraries, 3) inadequate follow-up capacity, 4) artificial and non-physiological assay systems, 5) a lack of suitable animal models for specific diseases, and 6) unpredictable compound toxicity. [154]. Several HTS assays have been developed in the past years, aiming for a cancer-target therapy. The majority of the approaches used in the cancer field are based on HTS of chemical and natural compound libraries, with a primary focus on protein kinases, though challenges like drug resistance and toxicity persist. However, certain therapeutic targets remain underexplored, with the glycobiology field being a prime example. Several interesting approaches have been employed for glyco-specific HTS assays (**Table 1**), though their variety is still relatively limited compared to other targets. Indeed, compared to other molecular targets, these analyses are still in a small number and lack further validation and clinical studies to be considered therapeutic possibilities [132].

Early HTS methods involved radioisotopes [155], which are now largely outdated. The introduction of fluorescence-based HTS assays has opened a world of possibilities for drug discovery, eliminating the need for radioactivity. Thus, the HTS fluorescence methods for glycobiological targets are now more common, due to their high sensitivity [156]. The most used is fluorescence polarization [157-160]. Nevertheless, numerous fluorescence techniques are available and are open to various possibilities and strategies. For example, Gross and colleagues proposed a FRET-based HTS to discover inhibitors that target OGT [161]. In 2013, another sensitive assay using fluorescence detection of UDP, GDP, and

CMP was developed [162], while in 2019, Zhang and colleagues introduced a glycosidase-coupled enzyme method using fluorogenically labeled oligosaccharides [156]. Furthermore, other non-fluorescent methods have been adapted to HTS. For instance, an enzyme-linked lectin assay (ELLA) approach was used to identify small molecule inhibitors of ppGalNAc-T1 [163], and a noncovalent carbohydrate microarray was used to find fucosyltransferase inhibitors [164].

Most of these HTS assays are biochemical comprising only targeted molecules and, this way, are missing the complexity of a cell or living system. They often result in higher hit numbers, however, these require further validation in cells or *in vivo* models, as these approaches do not account for the compound effect on the cell itself and in the whole 'system'. Therefore, cell-based assays, which can directly yield biologically effective hits, could improve the search for new therapies. These assays not only help evaluate therapeutic efficacy but also provide valuable information on the toxicity and specificity of candidate compounds, something biochemical assays cannot offer [132]. Moreover, with advances in gene-editing technologies, it is possible to engineer mammalian cells for cell-based screenings [165,166]. The first use of glycoengineered cell models for inhibitor screens was reported by Linstedt and colleagues, who used fluorescence biosensors for specific GalNAc-T isoenzymes to screen for isoenzyme-specific inhibitors. More specifically, a ppGalNAc-T3 biosensor was used to screen over 20,000 compounds, identifying one compound, T3Inh-1, that selectively blocked ppGalNAc-T3 activity without causing toxic side effects [167]. More recently, Clausen's group conducted an HTS of a small library using glycoengineered cells to selectively target ppGalNAc-T3. While two compounds were found to broadly inhibit Golgi-localized glycosylation processes, a specific glycosyltransferase inhibitor was not obtained. Nonetheless, the assay was validated, and this strategy holds potential for future screening of larger compound libraries [168].

Table 1: Different HTS approaches targeting several glycosyltransferases or glycans. Adapted from [104]

HTS assay	Target(s)	Type of assay	Ref
Radiolabeled ligands			
Solid-phase glycosyltransferase assay	GlcNAc-T V and Core 2 GlcNAc-T	Biochemical	[155]
Scintillation proximity assay	FucTVII	Biochemical	[169]
Scintillation proximity assay	FucTVII	Biochemical	[170]
Fluorescent methods			
Fluorescence polarization	O-GlcNAc transferase (OGT)	Biochemical	[159]

FRET	O-GlcNAc transferase (OGT)	Biochemical	[161]
Fluorescence polarization	ST3GalIII, ST6GalI, ST3GalI, FUTVI and FUTVII	Biochemical	[157]
Fluorescence detection of UDP, GDP, and CMP	galactosyltransferase B4GALT1	Biochemical	[162]
Cell-based fluorescence sensor	ppGalNAc-T3	Cell-based	[167]
Fluorogenically labeled oligosaccharide	FUTVI (adapted to all FUT)	Biochemical	[156]
N-linked glycosylation reporter	N-linked glycosylation	Cell-based	[171]
Fluorescence polarization	β -Kdo glycosyltransferases (bacteria)	Biochemical	[160]
Cell-based fluorescence sensor	ppGalNAc-T11	Cell-based	[168]
Other methods			
Enzyme-linked lectin assay (ELLA)	ppGalNAcT-1	Biochemical	[163]
Noncovalent carbohydrate microarray	FUTVI	Biochemical	[164]

3.2. Fluorinated Monosaccharide Analogs

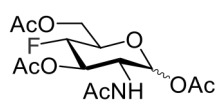
Strategies that manipulate glycosylation biosynthesis are emerging as promising approaches to target aberrant glycans in cancer. One example is the use of monosaccharide analogs [172-178]. By chemically introducing functional groups into the common monosaccharides, these modified sugars, which are misrecognized by the cell as the natural sugar, can disrupt glycans elongation. These can block the enzymatic addition of the natural monosaccharides to the glycan chain by competing against the natural donor, direct incorporation into the glycan chain, and/or interfering with the natural sugar donor biosynthetic conversion, all interfering with the normal glycosylation [173,179]. Over the years, numerous modifications and functional groups were introduced into different monosaccharides, including fluorine [173], azide [180], alkynyl [181], or thioester [182]. These analogs have shown significant promising results in blocking the biosynthesis of certain glycan structures and in tackling cancer cell malignancy. Among the various modifications, the introduction of one or more fluorine groups is a very common

monosaccharide modification. In fact, over the past two decades, fluorinated drugs have emerged as one of the fastest-growing classes of organic compounds. These pharmaceuticals have made up approximately 20% of commercialized medications nowadays [183]. In the field of glycoscience, fluorine was initially employed in probes to study glycan–protein interaction, specifically by using ^{18}F as a radiolabel tracer atom for positron emission tomography (PET) imaging [184]. In addition, 2-deoxy-2-[^{18}F]fluoro-D-glucose has become the most commonly used PET tracer in cancer diagnosis applications [185].

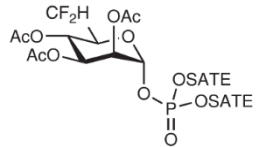
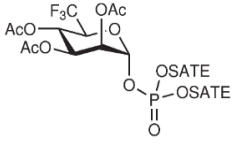
The synthesis of Fluorinated Monosaccharide Analogs (F-MAs) involves the chemical replacement of one or more hydroxyl groups (-OH) attached to the sugar carbons of natural monosaccharides with fluorine atoms (deoxyfluorination). This substitution was first reported last century and has continued to be explored to the present day [173,179,186-188]. Fluorine, being a small, hydrophobic and chemically inert atom, is ideal for translational studies. It is often used as a bioisostere of the OH- and H- groups, due to its similar size and polarity. Having a significant electronegativity, this atom destabilizes the oxocarbenium transition state in the reactions catalyzed by carbohydrate-binding enzymes, altering the enzyme activity [184]. The main challenge of using fluorinated analogs in a biological context is ensuring the efficient crossing of the cell membranes, which represents an important limitation of the drug's efficiency in living cells [173]. This obstacle can be addressed by the peracetylation of the F-MAs, a process that enhances cell permeability while also being easily and rapidly converted into the corresponding donor substrate within the cell [173,189].

The use of different F-MAs as glycan biosynthesis inhibitors to target aberrant glycans in cancer has shown promising preclinical results as a potential novel therapy in several types of cancers (**Table 2**).

Table 2: Overview of Fluorinated Monosaccharide Analogs (F-MAs) used in cancer research.

F-MAs	Structure	Glycans affected	Cancer cells tested	Refs
4F-GlcNAc		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction of LacNAc structures, SLeX, SLeA and <i>O</i>-GlcNAc levels; - Inhibition of GAG biosynthesis: HS, DS and CS	Ovarian Colon Melanoma Leukemia	[187,1 88,190 -192]

4F-GalNac		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction of LacNAc structures, SLeX, and <i>O</i>-GlcNAc levels; - Inhibition of GAG biosynthesis: DS and CS	Ovarian Leukemia Breast	[191,1 93]
6F-GalNac		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction <i>O</i>-GlcNAc levels; - Inhibition of GAG biosynthesis: HS, DS and CS	Ovarian	[191]
3F_{ax}-Neu5Ac		- Reduction of the general sialylation; - Increasing of overall fucosylation; - Abolishment of SLeX; - Reduction of Sialyl T antigen.	Melanoma Leukemia Neuroblastoma Cervical Prostate Pancreatic	[173,1 94- 198]
3F_{ax}-Neu5Ac-5-carbamate		- More potent than 3F_{ax}-Neu5Ac; - Reduction of the general sialylation.	Melanoma Leukemia, Neuroblastoma Cervical Prostate	[196,1 99]
2F-Fuc		- Reduction of the general fucosylation; - Reduction of core fucosylation; - Abolishment of SLeX and LeX.	Leukemia Liver Colon Cervical Lung Prostate	[199- 204]
2F-Fuc-1-P		- More potent than 2F-Fuc; - Reduction of the general fucosylation.	Leukemia Cervical Lung Prostate	[199,2 00]

Fucotrim I		▪ More potent than 2F-Fuc; ▪ Reduction of the general fucosylation; ▪ Increase of α2,6- and α2,3-linked sialic acids	Leukemia Prostate [199,201]
Fucotrim II		▪ More potent than 2F-Fuc; ▪ Reduction of the general fucosylation; ▪ Reduction of mannose <i>N</i>-glycans and α2,6- and α2,3-linked sialic acids;	Leukemia Prostate [199,201]

Overall, F-Neu5Ac and F-Fuc analogs are the most extensively studied F-MAs (**Table 2**). In 2012, Paulsen's lab introduced highly effective inhibitors of STs and FUTs: the peracetylated 3F_{ax}-Neu5Ac and 2F-Fuc, respectively. These analogs readily enter the cells and are converted into their fluorinated donor substrate counterparts, CMP-3F_{ax}-Neu5Ac or GDP-2F-Fuc. Through a dual-action mechanism, they inhibit enzymatic activity by competing with the natural Neu5Ac and Fuc, accumulating within the cell, and effectively halting the *de novo* synthesis of the substrate through a feedback loop due to their structural similarity to natural sugar substrates [173]. This groundbreaking study marked the beginning of increased research and development of these F-MAs, particularly in the field of cancer therapeutics.

3F_{ax}-Neu5Ac could successfully inhibit several STs, such as ST3GalI/II/IV/VI and ST6Gal enzymes, resulting in a remarkable reduction of the general sialylation [173]. This inhibition translated into effective biological outcomes, such as adhesion and migration in murine melanoma cells, showing no major cellular toxicity and strongly inhibiting tumor growth *in vivo* [194]. Moreover, the treatment affected the rolling in E-selectin and P-selectin coated surfaces, due to the abolishment of SLeX expression in leukocytes and melanoma cells [173]. Similar results were found in pancreatic cancer cells, where 3F_{ax}-Neu5Ac reduced tumor sialylation, E-selectin adhesion, migration, and invasion [197]. *In vivo* intratumorally injection of 3F_{ax}-Neu5Ac led to a reduction of tumor growth and increased tumor-infiltrating lymphocytes [195,197]. This analog also demonstrated efficacy in prostate cancer models, where pre-treatment inhibited tumor growth and bone metastasis [205]. Furthermore, 3F_{ax}-Neu5Ac was shown to significantly alter the tumor microenvironment and enhance tumor immunity by increasing tumor-infiltrating natural killer cells and CD8+ T-cells while reducing regulatory T-cells and myeloid-derived suppressor cells [195].

Despite these promising results, *in vivo* systemic administration of 3F_{ax}-Neu5Ac has limitations, causing mice renal failure [206]. To overcome this, Büll and colleagues developed a targeted delivery system by encapsulating the 3F_{ax}-Neu5Ac into tumor-targeting nanoparticles. Therefore, this analog was specifically delivered to melanoma cells *in vivo*, preventing the outgrowth of melanoma cells at distant sites while limiting exposure to normal cells [198]. Additionally, a recent alternative involves the synthesis of a photoactivatable 3F_{ax}-Neu5Ac derivative by incorporating a photolabile ortho-nitrobenzyl group. This allows for inducible and local inhibition of sialylated glycan biosynthesis in target cells or tissues, minimizing damage in healthy tissues [207]. Further modifications, including the addition of a carbamate group to the C5 position, enhanced 3F_{ax}-Neu5Ac metabolic stability and inhibitory capacity, resulting in prolonged and more effective inhibition in both mouse and human cancer and non-cancer cell lines [196,199].

Regarding 2F-Fuc, it was demonstrated as efficient in inhibiting FUT activity by targeting FUTIV, VII, and VIII enzymes, leading to the abolishment of SLeX and LeX and a reduction in core fucosylation [173]. Both *in vitro* and *in vivo* studies showed promising anti-cancer effects of orally bioavailable 2F-Fuc across various tumor types [173,202-204]. In fact, 2F-Fuc entered a Phase I clinical trial (NCT 02952989) for patients with advanced solid tumors, where it demonstrated potential in suppressing fucosylation and tumor growth. However, the trial was halted due to associated thromboembolic events [208]. Inspired by 2F-Fuc's efficacy, several additional fucosylation inhibitors have been developed [179]. For instance, a 2F-Fuc phosphate derivative, with a phosphate group added at the C1 position, showed increased potency and prolonged inhibitory effects compared to the original 2F-Fuc [199,200]. Also, inhibitors derived from fluorinated rhamnose 1-phosphate (Fucotrim I and II) effectively reduce cellular fucosylation [201]. These are converted into GDP-Man derivatives within the cell, reducing the levels of GDP-Man and competitively inhibiting the GDP-Man dehydratase (GMDS), an enzyme crucial for the *de novo* biosynthesis of GDP-Fuc. Cells treated with Fucotrim I and II have been shown to inhibit fucosylated glycans on the cell surface, impacting cell proliferation, viability, and colony formation in prostate cancer cells, while sparing normal prostate epithelium cells [199,201].

Besides F-Neu5Ac and F-Fuc, fluorinated HexNAc, that includes GlcNAc and GalNAc monosaccharides, were among the first to be synthesized, with GlcNAc having a principal focus. In the 1990s, Woynarowska and colleagues introduced 4F-GlcNAc as a glycan inhibitor that reduced the expression of terminal LacNAc, SLeX and SLeA structures, thereby hindering cell adhesion in ovarian cancer and CRC cell lines [187,188]. Later, peracetylated 4F-GlcNAc was shown to impair the expression of selectin ligands, such SLeX, and inhibit cell adhesion in leukemia cancer cells. In the same study, it was shown that this analog is not incorporated into *N*- or *O*-glycans. Instead, similar to 3F_{ax}-Neu5Ac

and 2F-Fuc, after entering the cell, 4F-GlcNAc is readily metabolized into UDP-4F-GlcNAc, which decreases the levels of the natural nucleotide sugars UDP-GlcNAc and UDP-GalNAc through a negative feedback mechanism [192]. In a mouse model of attenuated skin inflammatory reaction, 4F-GlcNAc reduced E- and P-selectin ligand biosynthesis and prevented leukocyte migration [209]. Additionally, *in vivo* administration of 4F-GlcNAc attenuated tumor growth in mice, and reduced galectin-1 ligand synthesis and galectin-1 mediated immune evasion. In fact, 4F-GlcNAc treatment led to an increase of effector T cells and a decrease of IL-10⁺T cells in galectin-1 expressing tumors. This suggests that 4F-GlcNAc could limit tumor growth by enhancing T cell-mediated immunity [190,210]. Moreover, 4F-GlcNAc was also shown to inhibit protein O-GlcNAc levels, similar to peracetylated 4F- and 6F-GalNAc [191]. While GalNAc analogs also exhibit potential in disrupting glycan biosynthesis and their biological functions, less research has focused on GalNAc analogs than other F-MAs. For instance, human leucocyte cells treated with peracetylated 4F-GalNAc showed reduced leukocyte selectin-mediated adhesion and migration to sites of inflammation due to SLeX inhibition [193]. Apart from N- and O-glycans, peracetylated 4F-GalNAc affects GAG biosynthesis, except for HS structures. On the other hand, peracetylated 6F-GalNAc drastically reduces all GAGs expression (CS, DS and HS), as well as of N- and O-linked glycans. Moreover, 6F-GalNAc treatment, using the *in vivo* chick embryo chorioallantoic membrane (CAM) model, decreased growth factor signaling and angiogenesis [191]. For 6F-GalNAc, its effects in glycosylation are due to the reduction of cellular levels of both UDP-GalNAc and UDP-GlcNAc, and there is no incorporation into glycan chains. As for 4F-GalNAc, Marante and colleagues showed that 4F-GalNAc can be incorporated into glycans structures of multiple cellular glycoproteins, including P-selectin glycoprotein ligand-1 (PSGL-1), impairing their normal glycosylation [193]. However, other studies suggested that UDP-4F-GalNAc acts by inhibiting the production of endogenous UDP-GalNAc/GlcNAc [211], and also by inducing a slight decrease of CMP-Neu5Ac and GDP-Man [180], further empathizing their role in sugar donor depletion. Nevertheless, more studies are needed to fully understand the mechanism behind these F-MAs, especially for 4F-GalNAc. Further modifications of 4F-GalNAc, as well as to 3F-GalNAc structure were also done, such as 1-O-deacetylation, which showed an increased efficacy in inhibiting the proliferation of human prostate cancer cells, even when compared to common chemotherapeutic agents: cisplatin and 5-FU [212]. The chemical addition of an organometallic moiety at the C2 position of 4F-GalNAc also showed increased cytotoxicity against cisplatin-resistant breast and ovarian cancer cells [213].

Thus, continuing research and improving strategies that can target cancer-associated glycans and their associated malignant properties will open opportunities for developing novel and more effective cancer therapies.

4. Aims and thesis outline

Cancer remains one of the deadliest diseases worldwide. In most cases, surgery, chemo- and radiotherapy are the only treatments given to patients. Target therapies, such as specific antibodies or small drug molecules (e.g., receptor kinase inhibitors), are limited to a specific group of patients. However, over time these treatments often result in side effects and resistance. This underscores the urgent need for new and more effective therapeutic strategies. Exploring different and poorly explored targets, such as cancer-associated glycans, holds significant potential in cancer therapy, providing novel treatment avenues.

Altered glycosylation, such as increased sialylation, is a feature in cancer and is primarily caused by dysregulation in the expression of GTs. Aberrant glycans have been shown to play crucial roles in several malignant properties of cancer cells. As an example and focus on this thesis, SLeX, a sialylated Lewis antigen commonly found overexpressed in cancer, has a crucial role in cancer progression and aggressiveness, being linked with metastasis formation and a worse patient prognosis. Notably, reducing or abolishing SLeX biosynthesis has shown promising results in limiting metastatic behavior. As a result, developing strategies that inhibit the biosynthesis of SLeX, for instance, by targeting associated GTs, offers a promising therapeutic approach. Furthermore, a deeper understanding of the specific GTs involved in the synthesis of these aberrant glycans can help improve the design of therapies that are more selective and effective in targeting cancer cells.

Therefore, in this thesis, we defined two main goals (**Fig 13**):

- 1) To elucidate the main STs involved in the biosynthesis of SLeX and its impact on the malignant properties of GIC cells;
- 2) To identify novel therapeutic molecules that target cancer-associated glycan biosynthesis in GIC cells;

The first goal, detailed in **Chapter 2**, focused on clarifying the role of ST3GalIV in SLeX biosynthesis in GIC cells. Using a CRISPR-Cas9 technology, our findings highlighted this enzyme as the main ST responsible for the biosynthesis of SLeX and its importance for cancer motility. However, we also acknowledged the involvement of other STs, such as ST3GalVI, in the biosynthesis of this glycan.

As for the second goal, where we proposed to identify novel therapeutic molecules that target cancer-associated glycans, we employed two different strategies which are described in detail in Chapter 3 and Chapter 4.

In **Chapter 3**, we established a fluorescent HTS method to identify compounds that inhibit the biosynthesis of SLeX expression in cancer cells. Through this approach, we

identified monensin as a promising SLeX inhibitor. We explored its mechanism of action and showed monensin's ability to reduce viability, motility and invasive capacity of SLeX-positive GIC cells. Using various *in vivo* models, including a pre-clinical organoid model, we further demonstrated monensin's capability to reduce tumor formation and invasion.

In **Chapter 4**, we evaluated the impact of different fluorinated GalNAc analogs on the biosynthesis of cancer-associated O-glycans. We have shown that only the GalNAc analog fluorinated at carbon 4, the 4F-GalNAc, effectively reduced the expression of both O-linked SLeX and STn. Furthermore, we identified altered genes involved in sugar donor conversion and *N*-glycan biosynthesis. Treatment with 4F-GalNAc impacted cell migration, invasion and E-selectin binding, and led to alterations of tyrosine kinase receptors in GIC cells. Furthermore, we have shown that the treatment with 4F-GalNAc led to a significant reduction in tumor growth in an *in vivo* mice model.

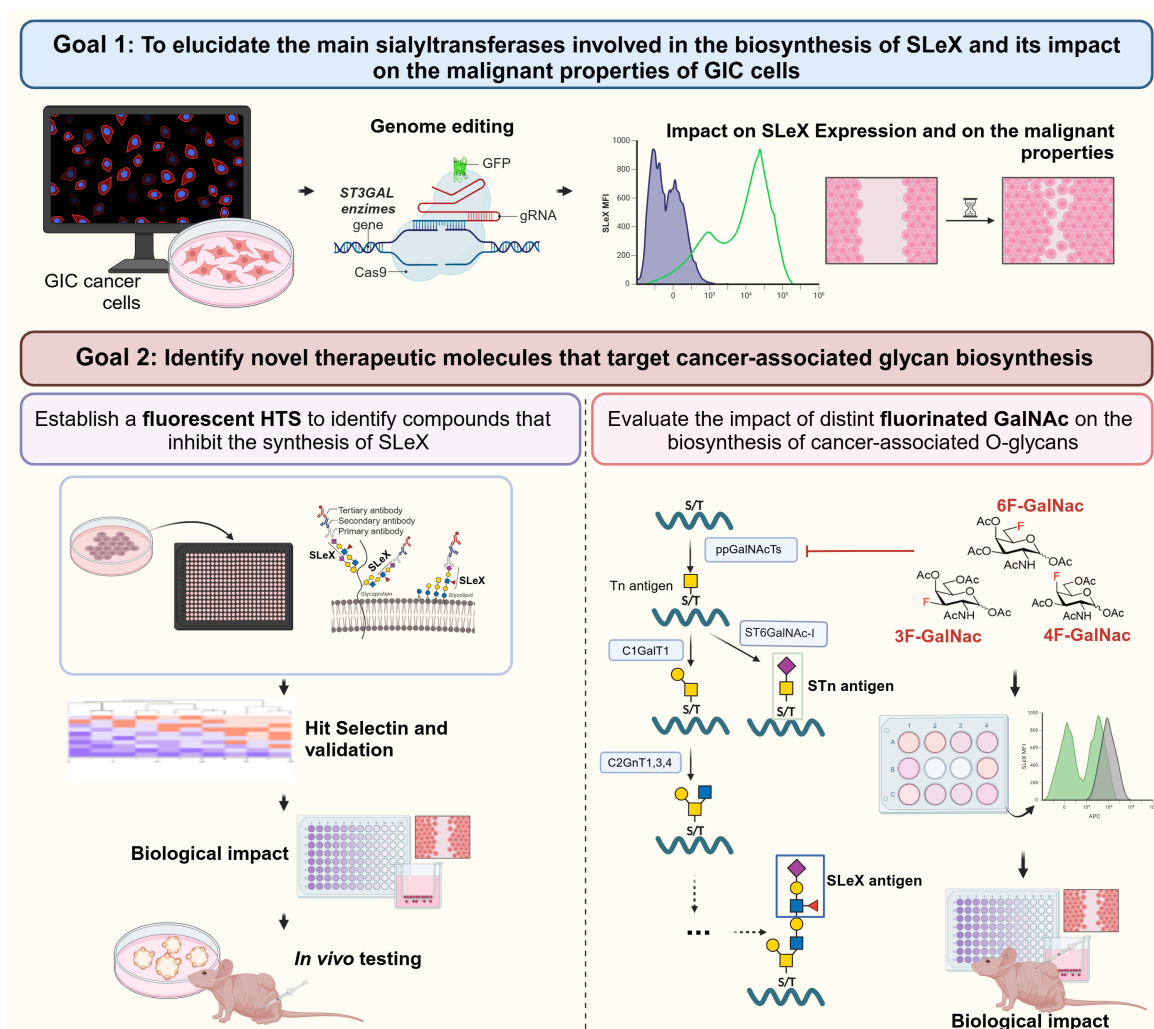


Figure 13: Main goals of this thesis. The first goal is to elucidate the role of ST3GalIV in the biosynthesis of SLeX and its associated motility capacities in GIC cells through the use of CRISPR-Cas9 gene editing. The second objective focuses on identifying novel therapeutic molecules that target cancer-associated glycans. To achieve this, we employ two different strategies: a fluorescence-based HTS approach, and the use of fluorinated GalNAc analogs.

5. References:

- [1] Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2024 May-Jun;74(3):229-263.
- [2] Dillekas H, Rogers MS, Straume O. Are 90% of deaths from cancer caused by metastases? *Cancer Med*. 2019 Sep;8(12):5574-5576.
- [3] Floor SL, Dumont JE, Maenhaut C, et al. Hallmarks of cancer: of all cancer cells, all the time? *Trends Mol Med*. 2012 Sep;18(9):509-15.
- [4] Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov*. 2022 Jan;12(1):31-46.
- [5] Morgado-Diaz JA. Gastrointestinal Cancers. In: Morgado-Diaz JA, editor. *Gastrointestinal Cancers*. Brisbane (AU)2022.
- [6] Jiao Y, Cheng Z, Gao Y, et al. Development and status quo of digestive endoscopy in China: An analysis based on the national census in 2013 and 2020. *J Transl Int Med*. 2024 Apr;12(2):177-187.
- [7] Smyth EC, Nilsson M, Grabsch HI, et al. Gastric cancer. *Lancet*. 2020 Aug 29;396(10251):635-648.
- [8] Sitarz R, Skierucha M, Mielko J, et al. Gastric cancer: epidemiology, prevention, classification, and treatment. *Cancer Manag Res*. 2018 Feb;10:239-248.
- [9] Polk DB, Peek RM, Jr. *Helicobacter pylori*: gastric cancer and beyond. *Nat Rev Cancer*. 2010 Jun;10(6):403-14.
- [10] Sexton RE, Al Hallak MN, Diab M, et al. Gastric cancer: a comprehensive review of current and future treatment strategies. *Cancer Metastasis Rev*. 2020 Dec;39(4):1179-1203.
- [11] Zhang R, Li H, Li N, et al. Risk factors for gastric cancer: a large-scale, population-based case-control study. *Chin Med J (Engl)*. 2021 Jul 22;134(16):1952-1958.
- [12] Vogelaar IP, van der Post RS, Bisseling TM, et al. Familial gastric cancer: detection of a hereditary cause helps to understand its etiology. *Hered Cancer Clin Pract*. 2012 Dec 12;10(1):18.
- [13] Cislo M, Filip AA, Arnold Offerhaus GJ, et al. Distinct molecular subtypes of gastric cancer: from Lauren to molecular pathology. *Oncotarget*. 2018 Apr 10;9(27):19427-19442.
- [14] Lauren P. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand*. 1965;64:31-49.

- [15] Hwang SW, Lee DH, Lee SH, et al. Preoperative staging of gastric cancer by endoscopic ultrasonography and multidetector-row computed tomography. *J Gastroenterol Hepatol*. 2010 Mar;25(3):512-8.
- [16] Polkowski W, van Sandick JW, Offerhaus GJ, et al. Prognostic value of Lauren classification and c-erbB-2 oncogene overexpression in adenocarcinoma of the esophagus and gastroesophageal junction. *Ann Surg Oncol*. 1999 Apr-May;6(3):290-7.
- [17] Parsonnet J, Vandersteen D, Goates J, et al. Helicobacter pylori infection in intestinal- and diffuse-type gastric adenocarcinomas. *J Natl Cancer Inst*. 1991 May 1;83(9):640-3.
- [18] Caldas C, Carneiro F, Lynch HT, et al. Familial gastric cancer: overview and guidelines for management. *J Med Genet*. 1999 Dec;36(12):873-80.
- [19] Carmo GCD, Cavalcante RM, Aquino TMF. Gastric cancer: an overview. *Rev Assoc Med Bras (1992)*. 2024 Jun;70(suppl 1):e2024S116.
- [20] Correa P, Piazuelo MB. The gastric precancerous cascade. *J Dig Dis*. 2012 Jan;13(1):2-9.
- [21] Zhao H, Hu H, Chen B, et al. Overview on the Role of E-Cadherin in Gastric Cancer: Dysregulation and Clinical Implications. *Front Mol Biosci*. 2021 Jun;8:689139.
- [22] Monster JL, Kemp LJS, Gloerich M, et al. Diffuse gastric cancer: Emerging mechanisms of tumor initiation and progression. *Biochim Biophys Acta Rev Cancer*. 2022 May;1877(3):188719.
- [23] Correa P. Gastric cancer: overview. *Gastroenterol Clin North Am*. 2013 Jun;42(2):211-7.
- [24] Cancer Genome Atlas Research N. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*. 2014 Sep 11;513(7517):202-9.
- [25] Strand MS, Lockhart AC, Fields RC. Genetics of Gastric Cancer. *Surg Clin North Am*. 2017 Apr;97(2):345-370.
- [26] Lordick F, Carneiro F, Cascinu S, et al. Gastric cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2022 Oct;33(10):1005-1020.
- [27] Locker GY, Hamilton S, Harris J, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol*. 2006 Nov 20;24(33):5313-27.
- [28] Reis CA, Osorio H, Silva L, et al. Alterations in glycosylation as biomarkers for cancer detection. *J Clin Pathol*. 2010 Apr;63(4):322-9.

- [29] Ferreira JA, Magalhaes A, Gomes J, et al. Protein glycosylation in gastric and colorectal cancers: Toward cancer detection and targeted therapeutics. *Cancer Lett.* 2017 Feb 28;387:32-45.
- [30] Matsuoka T, Yashiro M. Biomarkers of gastric cancer: Current topics and future perspective. *World J Gastroenterol.* 2018 Jul 14;24(26):2818-2832.
- [31] Takahashi Y, Takeuchi T, Sakamoto J, et al. The usefulness of CEA and/or CA19-9 in monitoring for recurrence in gastric cancer patients: a prospective clinical study. *Gastric Cancer.* 2003;6(3):142-5.
- [32] Nakagoe T, Sawai T, Tsuji T, et al. Pre-operative serum levels of sialyl Tn antigen predict liver metastasis and poor prognosis in patients with gastric cancer. *Eur J Surg Oncol.* 2001 Dec;27(8):731-9.
- [33] Li JK, Zheng M, Miao CW, et al. Peritoneal lavage cytology and carcinoembryonic antigen determination in predicting peritoneal metastasis and prognosis of gastric cancer. *World J Gastroenterol.* 2005 Dec 14;11(46):7374-7.
- [34] Glimelius B, Ekstrom K, Hoffman K, et al. Randomized comparison between chemotherapy plus best supportive care with best supportive care in advanced gastric cancer. *Ann Oncol.* 1997 Feb;8(2):163-8.
- [35] Van Cutsem E, Bang YJ, Feng-Yi F, et al. HER2 screening data from ToGA: targeting HER2 in gastric and gastroesophageal junction cancer. *Gastric Cancer.* 2015 Jul;18(3):476-84.
- [36] Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet.* 2010 Aug 28;376(9742):687-97.
- [37] Zhang J, Qiu W, Zhang W, et al. Tracking of trastuzumab resistance in patients with HER2-positive metastatic gastric cancer by CTC liquid biopsy. *Am J Cancer Res.* 2023;13(11):5684-5697.
- [38] Fuchs CS, Tomasek J, Yong CJ, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet.* 2014 Jan 4;383(9911):31-39.
- [39] Wilke H, Muro K, Van Cutsem E, et al. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol.* 2014 Oct;15(11):1224-35.
- [40] Fuchs CS, Doi T, Jang RW, et al. Safety and Efficacy of Pembrolizumab Monotherapy in Patients With Previously Treated Advanced Gastric and

- Gastroesophageal Junction Cancer: Phase 2 Clinical KEYNOTE-059 Trial. *JAMA Oncol.* 2018 May 10;4(5):e180013.
- [41] Paschke S, Jafarov S, Staib L, et al. Are Colon and Rectal Cancer Two Different Tumor Entities? A Proposal to Abandon the Term Colorectal Cancer. *Int J Mol Sci.* 2018 Aug 30;19(9).
 - [42] Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin.* 2024 Jan-Feb;74(1):12-49.
 - [43] Kuipers EJ, Grady WM, Lieberman D, et al. Colorectal cancer. *Nat Rev Dis Primers.* 2015 Nov 5;1:15065.
 - [44] Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol.* 2019 Dec;16(12):713-732.
 - [45] Marmol I, Sanchez-de-Diego C, Pradilla Dieste A, et al. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci.* 2017 Jan 19;18(1).
 - [46] Sawicki T, Ruszkowska M, Danielewicz A, et al. A Review of Colorectal Cancer in Terms of Epidemiology, Risk Factors, Development, Symptoms and Diagnosis. *Cancers (Basel).* 2021 Apr 22;13(9).
 - [47] Schmitt M, Greten FR. The inflammatory pathogenesis of colorectal cancer. *Nat Rev Immunol.* 2021 Oct;21(10):653-667.
 - [48] Singh MP, Rai S, Pandey A, et al. Molecular subtypes of colorectal cancer: An emerging therapeutic opportunity for personalized medicine. *Genes Dis.* 2021 Mar;8(2):133-145.
 - [49] Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med.* 2015 Nov;21(11):1350-6.
 - [50] Fessler E, Medema JP. Colorectal Cancer Subtypes: Developmental Origin and Microenvironmental Regulation. *Trends Cancer.* 2016 Sep;2(9):505-518.
 - [51] Cervena K, Siskova A, Buchler T, et al. Methylation-Based Therapies for Colorectal Cancer. *Cells.* 2020 Jun 24;9(6).
 - [52] Jelski W, Mroczko B. Biochemical Markers of Colorectal Cancer - Present and Future. *Cancer Manag Res.* 2020 Jun;12:4789-4797.
 - [53] Ogunwobi OO, Mahmood F, Akingboye A. Biomarkers in Colorectal Cancer: Current Research and Future Prospects. *Int J Mol Sci.* 2020 Jul 27;21(15).
 - [54] Argiles G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* 2020 Oct;31(10):1291-1305.

- [55] Andre T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol*. 2009 Jul 1;27(19):3109-16.
- [56] Cervantes A, Adam R, Rosello S, et al. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2023 Jan;34(1):10-32.
- [57] Adebayo AS, Agbaje K, Adesina SK, et al. Colorectal Cancer: Disease Process, Current Treatment Options, and Future Perspectives. *Pharmaceutics*. 2023 Nov 12;15(11).
- [58] Xie YH, Chen YX, Fang JY. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct Target Ther*. 2020 Mar 20;5(1):22.
- [59] Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol*. 2010 Mar 1;28(7):1254-61.
- [60] Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015 Sep;15(9):540-55.
- [61] Varki A, Kornfeld S. Historical Background and Overview. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 1-20.
- [62] Gagneux P, Hennet T, Varki A. Biological Functions of Glycans. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 79-92.
- [63] Freeze HH, Boyce M, Zachara NE, et al. Glycosylation Precursors. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 53-66.
- [64] Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta*. 1999 Dec 6;1473(1):4-8.
- [65] Stanley P, Moremen KW, Lewis NE, et al. N-Glycans. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 103-16.
- [66] Schjoldager KT, Narimatsu Y, Joshi HJ, et al. Global view of human protein glycosylation pathways and functions. *Nat Rev Mol Cell Biol*. 2020 Dec;21(12):729-749.
- [67] Brockhausen I, Wandall HH, Hagen KGT, et al. O-GalNAc Glycans. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 117-28.

- [68] Zachara NE, Akimoto Y, Boyce M, et al. The O-GlcNAc Modification. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 251-64.
- [69] Faria-Ramos I, Pocas J, Marques C, et al. Heparan Sulfate Glycosaminoglycans: (Un)Expected Allies in Cancer Clinical Management. *Biomolecules*. 2021 Jan 21;11(2).
- [70] Merry CLR, Lindahl U, Couchman J, et al. Proteoglycans and Sulfated Glycosaminoglycans. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 217-32.
- [71] Simpson M, Schaefer L, Hascall V, et al. Hyaluronan. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 205-16.
- [72] Komath SS FM, Hart GW, et al. Glycosylphosphatidylinositol Anchors. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology* [Internet]. 4th edition.: Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2022.
- [73] Schnaar RL SR, Tiemeyer M, et al. Glycosphingolipids. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology* [Internet]. 4th edition.: Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2022.
- [74] Flynn RA, Pedram K, Malaker SA, et al. Small RNAs are modified with N-glycans and displayed on the surface of living cells. *Cell*. 2021 Jun 10;184(12):3109-3124.e22.
- [75] Xie Y, Chai P, Till NA, et al. The modified RNA base acp(3)U is an attachment site for N-glycans in glycoRNA. *Cell*. 2024 Sep 19;187(19):5228-5237 e12.
- [76] Zhang N, Tang W, Torres L, et al. Cell surface RNAs control neutrophil recruitment. *Cell*. 2024 Feb 15;187(4):846-860 e17.
- [77] David L, Nesland JM, Clausen H, et al. Simple mucin-type carbohydrate antigens (Tn, sialosyl-Tn and T) in gastric mucosa, carcinomas and metastases. *APMIS Suppl*. 1992;27:162-72.
- [78] Dall'Olio F, Malagolini N, Trinchera M, et al. Mechanisms of cancer-associated glycosylation changes. *Front Biosci (Landmark Ed)*. 2012 Jan 1;17:670-99.
- [79] Reily C, Stewart TJ, Renfrow MB, et al. Glycosylation in health and disease. *Nat Rev Nephrol*. 2019 Jun;15(6):346-366.
- [80] Bellis SL, Reis CA, Varki A, et al. Glycosylation Changes in Cancer. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 631-44.
- [81] Mereiter S, Balmana M, Campos D, et al. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? *Cancer Cell*. 2019 Jul 8;36(1):6-16.

- [82] Stanley P, Wuhrer M, Lauc G, et al. Structures Common to Different Glycans. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 165-84.
- [83] Carvalho AS, Harduin-Lepers A, Magalhaes A, et al. Differential expression of alpha-2,3-sialyltransferases and alpha-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. *Int J Biochem Cell Biol*. 2010 Jan;42(1):80-9.
- [84] Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, et al. The human sialyltransferase family. *Biochimie*. 2001 Aug;83(8):727-37.
- [85] Padro M, Mejias-Luque R, Cobler L, et al. Regulation of glycosyltransferases and Lewis antigens expression by IL-1beta and IL-6 in human gastric cancer cells. *Glycoconj J*. 2011 Feb;28(2):99-110.
- [86] Somers WS, Tang J, Shaw GD, et al. Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. *Cell*. 2000 Oct 27;103(3):467-79.
- [87] Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun Signal*. 2008 Dec 4;6:10.
- [88] McEver RP. Selectin-carbohydrate interactions during inflammation and metastasis. *Glycoconj J*. 1997 Aug;14(5):585-91.
- [89] Zarbock A, Ley K, McEver RP, et al. Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow. *Blood*. 2011 Dec 22;118(26):6743-51.
- [90] Laubli H, Borsig L. Altered Cell Adhesion and Glycosylation Promote Cancer Immune Suppression and Metastasis. *Front Immunol*. 2019 Set;10:2120.
- [91] Tvaroska I, Selvaraj C, Koca J. Selectins-The Two Dr. Jekyll and Mr. Hyde Faces of Adhesion Molecules-A Review. *Molecules*. 2020 Jun 19;25(12).
- [92] Trinchera M, Aronica A, Dall'Olio F. Selectin Ligands Sialyl-Lewis a and Sialyl-Lewis x in Gastrointestinal Cancers. *Biology (Basel)*. 2017 Feb 23;6(1).
- [93] Nieswandt B, Hafner M, Echtenacher B, et al. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res*. 1999 Mar 15;59(6):1295-300.
- [94] Labelle M, Begum S, Hynes RO. Platelets guide the formation of early metastatic niches. *Proc Natl Acad Sci U S A*. 2014 Jul 29;111(30):E3053-61.
- [95] Tei K, Kawakami-Kimura N, Taguchi O, et al. Roles of cell adhesion molecules in tumor angiogenesis induced by cotransplantation of cancer and endothelial cells to nude rats. *Cancer Res*. 2002 Nov 1;62(21):6289-96.

- [96] Mathieu S, Gerolami R, Luis J, et al. Introducing alpha(1,2)-linked fucose into hepatocarcinoma cells inhibits vasculogenesis and tumor growth. *Int J Cancer*. 2007 Oct 15;121(8):1680-9.
- [97] Yin X, Rana K, Ponmudi V, et al. Knockdown of fucosyltransferase III disrupts the adhesion of circulating cancer cells to E-selectin without affecting hematopoietic cell adhesion. *Carbohydr Res*. 2010 Nov 2;345(16):2334-42.
- [98] Gomes C, Osorio H, Pinto MT, et al. Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS One*. 2013 Jun;8(6):e66737.
- [99] Shen L, Luo Z, Wu J, et al. Enhanced expression of alpha2,3-linked sialic acids promotes gastric cancer cell metastasis and correlates with poor prognosis. *Int J Oncol*. 2017 Apr;50(4):1201-1210.
- [100] Gomes C, Almeida A, Barreira A, et al. Carcinoembryonic antigen carrying SLe(X) as a new biomarker of more aggressive gastric carcinomas. *Theranostics*. 2019 Oct;9(24):7431-7446.
- [101] Numahata K, Satoh M, Handa K, et al. Sialosyl-Le(x) expression defines invasive and metastatic properties of bladder carcinoma. *Cancer*. 2002 Feb 1;94(3):673-85.
- [102] Feng H, Liang L, Deng W, et al. Sialyl Lewis X decorated integrin $\alpha 3$ on small extracellular vesicles promotes metastasis of bladder cancer via enhancing vascular permeability. *Angiogenesis*. 2024 Sep 2024/09/02.
- [103] Yoshihama N, Yamaguchi K, Chigita S, et al. A Novel Function of CD82/KAI1 in Sialyl Lewis Antigen-Mediated Adhesion of Cancer Cells: Evidence for an Anti-Metastasis Effect by Down-Regulation of Sialyl Lewis Antigens. *PLoS One*. 2015 Apr;10(4):e0124743.
- [104] Sakuma K, Aoki M, Kannagi R. Transcription factors c-Myc and CDX2 mediate E-selectin ligand expression in colon cancer cells undergoing EGF/bFGF-induced epithelial-mesenchymal transition. *Proc Natl Acad Sci U S A*. 2012 May 15;109(20):7776-81.
- [105] Guerrero PE, Miro L, Wong BS, et al. Knockdown of alpha2,3-Sialyltransferases Impairs Pancreatic Cancer Cell Migration, Invasion and E-selectin-Dependent Adhesion. *Int J Mol Sci*. 2020 Aug 28;21(17).
- [106] Thomas SN, Zhu F, Schnaar RL, et al. Carcinoembryonic antigen and CD44 variant isoforms cooperate to mediate colon carcinoma cell adhesion to E- and L-selectin in shear flow. *J Biol Chem*. 2008 Jun 6;283(23):15647-55.
- [107] Aarons CB, Bajenova O, Andrews C, et al. Carcinoembryonic antigen-stimulated THP-1 macrophages activate endothelial cells and increase cell-cell adhesion of colorectal cancer cells. *Clin Exp Metastasis*. 2007 May;24(3):201-9.

- [108] Bramswig KH, Poettler M, Unseld M, et al. Soluble carcinoembryonic antigen activates endothelial cells and tumor angiogenesis. *Cancer Res.* 2013 Nov 15;73(22):6584-96.
- [109] Toth CA, Thomas P, Broitman SA, et al. Receptor-mediated endocytosis of carcinoembryonic antigen by rat liver Kupffer cells. *Cancer Res.* 1985 Jan;45(1):392-7.
- [110] Bajenova OV, Zimmer R, Stolper E, et al. Heterogeneous RNA-binding protein M4 is a receptor for carcinoembryonic antigen in Kupffer cells. *J Biol Chem.* 2001 Aug 17;276(33):31067-73.
- [111] Gangopadhyay A, Bajenova O, Kelly TM, et al. Carcinoembryonic antigen induces cytokine expression in Kupffer cells: implications for hepatic metastasis from colorectal cancer. *Cancer Res.* 1996 Oct 15;56(20):4805-10.
- [112] Gangopadhyay A, Lazure DA, Thomas P. Carcinoembryonic antigen induces signal transduction in Kupffer cells. *Cancer Lett.* 1997 Sep 16;118(1):1-6.
- [113] Freitas D, Campos D, Gomes J, et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. *EBioMedicine.* 2019 Feb;40:349-362.
- [114] Karlen P, Young E, Brostrom O, et al. Sialyl-Tn antigen as a marker of colon cancer risk in ulcerative colitis: relation to dysplasia and DNA aneuploidy. *Gastroenterology.* 1998 Dec;115(6):1395-404.
- [115] Itzkowitz SH, Yuan M, Montgomery CK, et al. Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. *Cancer Res.* 1989 Jan 1;49(1):197-204.
- [116] Marcos NT, Bennett EP, Gomes J, et al. ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues. *Front Biosci (Elite Ed).* 2011 Jun 1;3:1443-55.
- [117] Radhakrishnan P, Dabelsteen S, Madsen FB, et al. Immature truncated O-glycophenotype of cancer directly induces oncogenic features. *Proc Natl Acad Sci U S A.* 2014 Sep 30;111(39):E4066-75.
- [118] Sewell R, Backstrom M, Dalziel M, et al. The ST6GalNAc-I sialyltransferase localizes throughout the Golgi and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. *J Biol Chem.* 2006 Feb 10;281(6):3586-94.
- [119] Lottich SC, Johnston WW, Szpak CA, et al. Tumor-associated antigen TAG-72: correlation of expression in primary and metastatic breast carcinoma lesions. *Breast Cancer Res Treat.* 1985;6(1):49-56.
- [120] Lima L, Neves M, Oliveira MI, et al. Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. *Urol Oncol.* 2017 Dec;35(12):675 e1-675 e8.

- [121] Munkley J. The Role of Sialyl-Tn in Cancer. *Int J Mol Sci.* 2016 Feb 24;17(3):275.
- [122] Ju T, Lanneau GS, Gautam T, et al. Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. *Cancer Res.* 2008 Mar 15;68(6):1636-46.
- [123] Gill DJ, Clausen H, Bard F. Location, location, location: new insights into O-GalNAc protein glycosylation. *Trends Cell Biol.* 2011 Mar;21(3):149-58.
- [124] Thomas D, Sagar S, Caffrey T, et al. Truncated O-glycans promote epithelial-to-mesenchymal transition and stemness properties of pancreatic cancer cells. *J Cell Mol Med.* 2019 Oct;23(10):6885-6896.
- [125] Julien S, Adriaenssens E, Ottenberg K, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. *Glycobiology.* 2006 Jan;16(1):54-64.
- [126] Julien S, Picco G, Sewell R, et al. Sialyl-Tn vaccine induces antibody-mediated tumour protection in a relevant murine model. *Br J Cancer.* 2009 Jun 2;100(11):1746-54.
- [127] Ozaki H, Matsuzaki H, Ando H, et al. Enhancement of metastatic ability by ectopic expression of ST6GalNAc on a gastric cancer cell line in a mouse model. *Clin Exp Metastasis.* 2012 Mar;29(3):229-38.
- [128] Campos D, Freitas D, Gomes J, et al. Probing the O-glycoproteome of gastric cancer cell lines for biomarker discovery. *Mol Cell Proteomics.* 2015 Jun;14(6):1616-29.
- [129] Gaiteiro C, Soares J, Relvas-Santos M, et al. Glycoproteogenomics characterizes the CD44 splicing code associated with bladder cancer invasion. *Theranostics.* 2022 Mar;12(7):3150-3177.
- [130] Clement M, Rocher J, Loirand G, et al. Expression of sialyl-Tn epitopes on beta1 integrin alters epithelial cell phenotype, proliferation and haptotaxis. *J Cell Sci.* 2004 Oct 1;117(Pt 21):5059-69.
- [131] Kariya Y, Kanno M, Matsumoto-Morita K, et al. Osteopontin O-glycosylation contributes to its phosphorylation and cell-adhesion properties. *Biochem J.* 2014 Oct 1;463(1):93-102.
- [132] Costa AF, Campos D, Reis CA, et al. Targeting Glycosylation: A New Road for Cancer Drug Discovery. *Trends Cancer.* 2020 Sep;6(9):757-766.
- [133] Arlotta KJ, Owen SC. Antibody and antibody derivatives as cancer therapeutics. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2019 Sep;11(5):e1556.
- [134] Vallinayagam L, Aldil.A, Ahmed N, et al. Small molecule inhibitors as emerging cancer therapeutics. *Integrative Cancer Science and Therapeutics.* 2014 Nov;1(3).
- [135] Duarte HO, Rodrigues JG, Gomes C, et al. ST6Gal1 targets the ectodomain of ErbB2 in a site-specific manner and regulates gastric cancer cell sensitivity to trastuzumab. *Oncogene.* 2021 May;40(21):3719-3733.

- [136] Lee HH, Wang YN, Xia W, et al. Removal of N-Linked Glycosylation Enhances PD-L1 Detection and Predicts Anti-PD-1/PD-L1 Therapeutic Efficacy. *Cancer Cell*. 2019 Aug 12;36(2):168-178 e4.
- [137] Sorensen AL, Reis CA, Tarp MA, et al. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. *Glycobiology*. 2006 Feb;16(2):96-107.
- [138] Rivalland G, Loveland B, Mitchell P. Update on Mucin-1 immunotherapy in cancer: a clinical perspective. *Expert Opin Biol Ther*. 2015 Oct;15(12):1773-87.
- [139] Berois N, Pittini A, Osinaga E. Targeting Tumor Glycans for Cancer Therapy: Successes, Limitations, and Perspectives. *Cancers (Basel)*. 2022 Jan 27;14(3).
- [140] Freitas R, Peixoto A, Ferreira E, et al. Immunomodulatory glycomedicine: Introducing next generation cancer glycovaccines. *Biotechnol Adv*. 2023 Jul-Aug;65:108144.
- [141] Freitas R, Miranda A, Ferreira D, et al. A multivalent CD44 glycoconjugate vaccine candidate for cancer immunotherapy. *J Control Release*. 2024 Mar;367:540-556.
- [142] Aasted MKM, Groen AC, Keane JT, et al. Targeting Solid Cancers with a Cancer-Specific Monoclonal Antibody to Surface Expressed Aberrantly O-glycosylated Proteins. *Mol Cancer Ther*. 2023 Oct 2;22(10):1204-1214.
- [143] Posey AD, Jr., Schwab RD, Boesteanu AC, et al. Engineered CAR T Cells Targeting the Cancer-Associated Tn-Glycoform of the Membrane Mucin MUC1 Control Adenocarcinoma. *Immunity*. 2016 Jun 21;44(6):1444-54.
- [144] Moghimi B, Muthugounder S, Jambon S, et al. Preclinical assessment of the efficacy and specificity of GD2-B7H3 SynNotch CAR-T in metastatic neuroblastoma. *Nature Communications*. 2021 Jan;12(1):511.
- [145] Sujjitjoon J, Sayour E, Tsao ST, et al. GD2-specific chimeric antigen receptor-modified T cells targeting retinoblastoma - assessing tumor and T cell interaction. *Transl Oncol*. 2021 Feb;14(2):100971.
- [146] Ritchie DS, Neeson PJ, Khot A, et al. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. *Mol Ther*. 2013 Nov;21(11):2122-9.
- [147] Abrantes R, Duarte HO, Gomes C, et al. CAR-Ts: new perspectives in cancer therapy. *FEBS Lett*. 2022 Feb;596(4):403-416.
- [148] Fu Z, Li S, Han S, et al. Antibody drug conjugate: the "biological missile" for targeted cancer therapy. *Signal Transduct Target Ther*. 2022 Mar 22;7(1):93.
- [149] Yang MC, Shia CS, Li WF, et al. Preclinical Studies of OBI-999: A Novel Globo H-Targeting Antibody-Drug Conjugate. *Mol Cancer Ther*. 2021 Jun;20(6):1121-1132.

- [150] Brassard J, Hughes MR, Roskelley CD, et al. Antibody-Drug Conjugates Targeting Tumor-Specific Mucin Glycoepitopes. *Front Biosci (Landmark Ed)*. 2022 Nov 4;27(11):301.
- [151] Al-Alem L, Prendergast JM, Clark J, et al. Sialyl-Tn serves as a potential therapeutic target for ovarian cancer. *J Ovarian Res*. 2024 Apr 2;17(1):71.
- [152] Xiao H, Woods EC, Vukojicic P, et al. Precision glycocalyx editing as a strategy for cancer immunotherapy. *Proc Natl Acad Sci U S A*. 2016 Sep 13;113(37):10304-9.
- [153] Fox S, Farr-Jones S, Sopchak L, et al. High-throughput screening: update on practices and success. *J Biomol Screen*. 2006 Oct;11(7):864-9.
- [154] Macarron R, Banks MN, Bojanic D, et al. Impact of high-throughput screening in biomedical research. *Nat Rev Drug Discov*. 2011 Mar;10(3):188-95.
- [155] Donovan RS, Datti A, Baek MG, et al. A solid-phase glycosyltransferase assay for high-throughput screening in drug discovery research. *Glycoconjugate Journal*. 1999 Oct;16(10):607-615.
- [156] Zhang X, Chen F, Petrella A, et al. A High-Throughput Glycosyltransferase Inhibition Assay for Identifying Molecules Targeting Fucosylation in Cancer Cell-Surface Modification. *ACS Chem Biol*. 2019 Apr 19;14(4):715-724.
- [157] Rillahan CD, Brown SJ, Register AC, et al. High-throughput screening for inhibitors of sialyl- and fucosyltransferases. *Angew Chem Int Ed Engl*. 2011 Dec 23;50(52):12534-7.
- [158] Hall MD, Yasgar A, Peryea T, et al. Fluorescence polarization assays in high-throughput screening and drug discovery: a review. *Methods Appl Fluoresc*. 2016 Apr 28;4(2):022001.
- [159] Gross BJ, Kraybill BC, Walker S. Discovery of O-GlcNAc transferase inhibitors. *J Am Chem Soc*. 2005 Oct 26;127(42):14588-9.
- [160] Gao Z, Ovchinnikova OG, Huang B-S, et al. High-Throughput "FP-Tag" Assay for the Identification of Glycosyltransferase Inhibitors. *Journal of the American Chemical Society*. 2019 Jan;141(6):2201-2204.
- [161] Gross BJ, Swoboda JG, Walker S. A strategy to discover inhibitors of O-linked glycosylation. *J Am Chem Soc*. 2008 Jan 16;130(2):440-1.
- [162] Kumagai K, Kojima H, Okabe T, et al. Development of a highly sensitive, high-throughput assay for glycosyltransferases using enzyme-coupled fluorescence detection. *Anal Biochem*. 2014 Feb 15;447:146-55.
- [163] Hang HC, Yu C, Ten Hagen KG, et al. Small molecule inhibitors of mucin-type O-linked glycosylation from a uridine-based library. *Chem Biol*. 2004 Mar;11(3):337-45.

- [164] Bryan MC, Lee LV, Wong CH. High-throughput identification of fucosyltransferase inhibitors using carbohydrate microarrays. *Bioorganic & Medicinal Chemistry Letters*. 2004 Jun 21;14(12):3185-3188.
- [165] Narimatsu Y, Joshi HJ, Nason R, et al. An Atlas of Human Glycosylation Pathways Enables Display of the Human Glycome by Gene Engineered Cells. *Mol Cell*. 2019 Jul 25;75(2):394-407 e5.
- [166] Narimatsu Y, Joshi HJ, Yang Z, et al. A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome. *Glycobiology*. 2018 May 1;28(5):295-305.
- [167] Song L, Linstedt AD. Inhibitor of ppGalNAc-T3-mediated O-glycosylation blocks cancer cell invasiveness and lowers FGF23 levels. *Elife*. 2017 Mar 31;6.
- [168] Sorensen DM, Bull C, Madsen TD, et al. Identification of global inhibitors of cellular glycosylation. *Nat Commun*. 2023 Feb 20;14(1):948.
- [169] Miyashiro M, Furuya S, Sugita T. A high-throughput screening system for alpha 1-3 fucosyltransferase-VII inhibitor utilizing scintillation proximity assay. *Analytical Biochemistry*. 2005 Mar 1;338(1):168-170.
- [170] Ahsen O, Voigtmann U, Klotz M, et al. A miniaturized high-throughput screening assay for fucosyltransferase VII. *Anal Biochem*. 2008 Jan 1;372(1):96-105.
- [171] Lopez-Sambrooks C, Shrimal S, Khodier C, et al. Oligosaccharyltransferase inhibition induces senescence in RTK-driven tumor cells. *Nat Chem Biol*. 2016 Dec;12(12):1023-1030.
- [172] Esko JD, Bertozzi C, Schnaar RL. Chemical Tools for Inhibiting Glycosylation. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 3rd ed. Cold Spring Harbor (NY)2015. p. 701-12.
- [173] Rillahan CD, Antonopoulos A, Lefort CT, et al. Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. *Nat Chem Biol*. 2012 Jul;8(7):661-8.
- [174] Martin KC, Tricomi J, Corzana F, et al. Fucosyltransferase-specific inhibition via next generation of fucose mimetics. *Chem Commun (Camb)*. 2021 Jan 28;57(9):1145-1148.
- [175] Afosah DK, Al-Horani RA. Sulfated Non-Saccharide Glycosaminoglycan Mimetics as Novel Drug Discovery Platform for Various Pathologies. *Curr Med Chem*. 2020 Jul;27(21):3412-3447.
- [176] Morla S, Ravikumar O, O'Hara C, et al. Designing Synthetic, Sulfated Glycosaminoglycan Mimetics That Are Orally Bioavailable and Exhibiting In Vivo Anticancer Activity. *J Med Chem*. 2023 Jan 26;66(2):1321-1338.

- [177] Li B, Kawatkar SP, George S, et al. Inhibition of Golgi mannosidase II with mannostatin A analogues: synthesis, biological evaluation, and structure-activity relationship studies. *Chembiochem*. 2004 Sep 6;5(9):1220-7.
- [178] Liu L, Prudden AR, Capicciotti CJ, et al. Streamlining the chemoenzymatic synthesis of complex N-glycans by a stop and go strategy. *Nat Chem*. 2019 Feb;11(2):161-169.
- [179] Rossing E, Pijnenborg JFA, Boltje TJ. Chemical tools to track and perturb the expression of sialic acid and fucose monosaccharides. *Chem Commun (Camb)*. 2022 Nov 1;58(87):12139-12150.
- [180] Del Solar V, Gupta R, Zhou Y, et al. Robustness in glycosylation systems: effect of modified monosaccharides, acceptor decoys and azido sugars on cellular nucleotide-sugar levels and pattern of N-linked glycosylation. *Mol Omics*. 2020 Aug 1;16(4):377-386.
- [181] Kizuka Y, Nakano M, Yamaguchi Y, et al. An Alkynyl-Fucose Halts Hepatoma Cell Migration and Invasion by Inhibiting GDP-Fucose-Synthesizing Enzyme FX, TSTA3. *Cell Chem Biol*. 2017 Dec 21;24(12):1467-1478 e5.
- [182] Zandberg WF, Kumarasamy J, Pinto BM, et al. Metabolic inhibition of sialyl-Lewis X biosynthesis by 5-thiofucose remodels the cell surface and impairs selectin-mediated cell adhesion. *J Biol Chem*. 2012 Nov 16;287(47):40021-30.
- [183] Shabir G, Saeed A, Zahid W, et al. Chemistry and Pharmacology of Fluorinated Drugs Approved by the FDA (2016-2022). *Pharmaceuticals (Basel)*. 2023 Aug 15;16(8).
- [184] Hevey R. Bioisosteres of Carbohydrate Functional Groups in Glycomimetic Design. *Biomimetics (Basel)*. 2019 Jul 28;4(3).
- [185] Coenen HH, Elsinga PH, Iwata R, et al. Fluorine-18 radiopharmaceuticals beyond [18F]FDG for use in oncology and neurosciences. *Nucl Med Biol*. 2010 Oct;37(7):727-40.
- [186] Sharma M, Bernacki RJ, Paul B, et al. Fluorinated carbohydrates as potential plasma membrane modifiers. Synthesis of 4- and 6-fluoro derivatives of 2-acetamido-2-deoxy-D-hexopyranoses. *Carbohydr Res*. 1990 May 1;198(2):205-21.
- [187] Woynarowska B, Skrincosky DM, Haag A, et al. Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. *J Biol Chem*. 1994 Sep 9;269(36):22797-803.
- [188] Woynarowska B, Dimitroff CJ, Sharma M, et al. Inhibition of human HT-29 colon carcinoma cell adhesion by a 4-fluoro-glucosamine analogue. *Glycoconj J*. 1996 Aug;13(4):663-74.

- [189] Gloster TM, Zandberg WF, Heinonen JE, et al. Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. *Nat Chem Biol.* 2011 Mar;7(3):174-81.
- [190] Cedeno-Laurent F, Opperman MJ, Barthel SR, et al. Metabolic inhibition of galectin-1-binding carbohydrates accentuates antitumor immunity. *J Invest Dermatol.* 2012 Feb;132(2):410-20.
- [191] van Wijk XM, Lawrence R, Thijssen VL, et al. A common sugar-nucleotide-mediated mechanism of inhibition of (glycosamino)glycan biosynthesis, as evidenced by 6F-GalNAc (Ac3). *FASEB J.* 2015 Jul;29(7):2993-3002.
- [192] Barthel SR, Antonopoulos A, Cedeno-Laurent F, et al. Peracetylated 4-fluoroglucosamine reduces the content and repertoire of N- and O-glycans without direct incorporation. *J Biol Chem.* 2011 Jun 17;286(24):21717-31.
- [193] Marathe DD, Buffone A, Jr., Chandrasekaran EV, et al. Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins. *Blood.* 2010 Feb 11;115(6):1303-12.
- [194] Bull C, Boltje TJ, Wassink M, et al. Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. *Mol Cancer Ther.* 2013 Oct;12(10):1935-46.
- [195] Bull C, Boltje TJ, Balneger N, et al. Sialic Acid Blockade Suppresses Tumor Growth by Enhancing T-cell-Mediated Tumor Immunity. *Cancer Res.* 2018 Jul 1;78(13):3574-3588.
- [196] Heise T, Pijnenborg JFA, Bull C, et al. Potent Metabolic Sialylation Inhibitors Based on C-5-Modified Fluorinated Sialic Acids. *J Med Chem.* 2019 Jan 24;62(2):1014-1021.
- [197] Miro L, Lopez J, Guerrero PE, et al. Sialyltransferase Inhibitor Ac(5)3F(ax)Neu5Ac Reverts the Malignant Phenotype of Pancreatic Cancer Cells, and Reduces Tumor Volume and Favors T-Cell Infiltrates in Mice. *Cancers (Basel).* 2022 Dec 12;14(24).
- [198] Bull C, Boltje TJ, van Dinther EA, et al. Targeted delivery of a sialic acid-blocking glycomimetic to cancer cells inhibits metastatic spread. *ACS Nano.* 2015 Jan 27;9(1):733-45.
- [199] Orozco-Moreno M, Visser EA, Hodgson K, et al. Targeting aberrant sialylation and fucosylation in prostate cancer cells using potent metabolic inhibitors. *Glycobiology.* 2023 Dec 30;33(12):1155-1171.
- [200] Pijnenborg JFA, Visser EA, Noga M, et al. Cellular Fucosylation Inhibitors Based on Fluorinated Fucose-1-phosphates*. *Chemistry.* 2021 Feb 24;27(12):4022-4027.
- [201] Pijnenborg JFA, Rossing E, Merx J, et al. Fluorinated rhamnosides inhibit cellular fucosylation. *Nat Commun.* 2021 Dec 2;12(1):7024.

- [202] Okeley NM, Alley SC, Anderson ME, et al. Development of orally active inhibitors of protein and cellular fucosylation. *Proc Natl Acad Sci U S A*. 2013 Apr 2;110(14):5404-9.
- [203] Zhou Y, Fukuda T, Hang Q, et al. Inhibition of fucosylation by 2-fluorofucose suppresses human liver cancer HepG2 cell proliferation and migration as well as tumor formation. *Sci Rep*. 2017 Sep 14;7(1):11563.
- [204] Carrascal MA, Silva M, Ramalho JS, et al. Inhibition of fucosylation in human invasive ductal carcinoma reduces E-selectin ligand expression, cell proliferation, and ERK1/2 and p38 MAPK activation. *Mol Oncol*. 2018 May;12(5):579-593.
- [205] Hodgson K, Orozco-Moreno M, Goode EA, et al. Sialic acid blockade inhibits the metastatic spread of prostate cancer to bone. *EBioMedicine*. 2024 Jun;104:105163.
- [206] Macauley MS, Arlian BM, Rillahan CD, et al. Systemic blockade of sialylation in mice with a global inhibitor of sialyltransferases. *J Biol Chem*. 2014 Dec 19;289(51):35149-58.
- [207] Moons SJ, Hornikx D, Aasted MKM, et al. UV light-induced spatial loss of sialic acid capping using a photoactivatable sialyltransferase inhibitor. *RSC Chem Biol*. 2023 Jul 5;4(7):506-511.
- [208] Do KT, Chow LQM, Reckamp K, et al. First-In-Human, First-In-Class, Phase I Trial of the Fucosylation Inhibitor SGN-2FF in Patients with Advanced Solid Tumors. *Oncologist*. 2021 Nov;26(11):925-e1918.
- [209] Dimitroff CJ, Kupper TS, Sackstein R. Prevention of leukocyte migration to inflamed skin with a novel fluorosugar modifier of cutaneous lymphocyte-associated antigen. *J Clin Invest*. 2003 Oct;112(7):1008-18.
- [210] Dimitroff CJ. Leveraging fluorinated glucosamine action to boost antitumor immunity. *Curr Opin Immunol*. 2013 Apr;25(2):206-13.
- [211] Jackson EG, Cutolo G, Yang B, et al. 4-Deoxy-4-fluoro-GalNAz (4FGalNAz) Is a Metabolic Chemical Reporter of O-GlcNAc Modifications, Highlighting the Notable Substrate Flexibility of O-GlcNAc Transferase. *ACS Chem Biol*. 2022 Jan 21;17(1):159-170.
- [212] Hornik S, Cervenková Stastná L, Curinova P, et al. Synthesis and in vitro cytotoxicity of acetylated 3-fluoro, 4-fluoro and 3,4-difluoro analogs of D-glucosamine and D-galactosamine. *Beilstein J Org Chem*. 2016 Apr;12:750-9.
- [213] Hamala V, Ondrášková K, Šťastná L, et al. Improving the anticancer activity of fluorinated glucosamine and galactosamine analogs by attachment of a ferrocene or ruthenium tetrazene motif. *Applied Organometallic Chemistry*. 2024 Feb;38(5):e7399.

Chapter 2

ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and associates with cancer cell motility

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ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and associates with cancer cell motility

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Abstract

Expression of sialyl Lewis X (SLeX) is a well-documented event during malignant transformation of cancer cells, and largely associates with their invasive and metastatic properties. Glycoproteins and glycolipids are the main carriers of SLeX, whose biosynthesis is known to be performed by different glycosyltransferases, namely by the family of β -galactoside- α 2,3-sialyltransferases (ST3Gals). In this study, we sought to elucidate the role of ST3GalIV in the biosynthesis of SLeX and in malignant properties of gastrointestinal (GI) cancer cells. By immunofluorescent screening, we selected SLeX-positive GI cancer cell lines and silenced ST3GalIV expression via CRISPR/Cas9. Flow cytometry, immunofluorescence and western blot analysis showed that ST3GalIV KO efficiently impaired SLeX expression in most cancer cell lines, with the exception of the colon cancer cell line LS174T. The impact of ST3GalIV KO in the biosynthesis of SLeX isomer SLeA and non sialylated Lewis X and A were also evaluated and overall, ST3GalIV KO led to a decreased expression of SLeA and an increased expression in both LeX and LeA. In addition, the abrogation of SLeX on GI cancer cells led to a reduction in cell motility. Furthermore, ST3GalVI KO was performed in LS174T ST3GalIV KO cells, resulting in the complete abolishment of SLeX expression and consequent reduced motility capacity of those cells. Overall, these findings portray ST3GalIV as the main, but not the only, enzyme driving the biosynthesis of SLeX in GI cancer cells, with a functional impact on cancer cell motility.

Keywords ST3GalIV · SLeX · Cancer cells · Cell motility

Introduction

Glycosylation is a major process that regulates several important features of all cells and plays a pivotal role in both health and disease [1, 2]. Through to the highly compartmentalized

and coordinated action of hundreds of enzymes, a myriad of glycan structures is synthesized and can be found attached to distinct molecules such as proteins and lipids [3]. Sialic acids, usually present at the terminal end of glycan structures, play crucial roles in cellular adhesion [4] and communication [5], antibody-dependent cellular cytotoxicity [6, 7], and cancer development and progression [8].

It is well established that cancer cells express aberrant profiles of cellular glycans [9]. For instance, increased expression of sialylated glycans, such as the sialylated Lewis antigens SLeA and SLeX, has been vastly documented in the majority of cancer types. Usually, the increased expression of these glycans is associated with enhanced cancer cell motility and invasion [9–11], and further translates, *in vivo*, to patient's poor survival [12]. In fact, the expression level of SLeA is routinely assessed through the CA19.9 serological test to infer cancer presence, recurrence, and treatment follow-up.

The increased expression of SLeA and SLeX in cancer is mainly due to the expression of enzymes involved in their intracellular biosynthesis, namely the sialyltransferases (STs). STs are a group of glycosyltransferases that catalyze

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the addition of sialic acids to the terminal non-reducing ends of glycan structures. This family comprises several enzymes with distinct specificities that were already comprehensively described by Harduin–Lepers *et al.* [13]. SLeX, Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc-R, is a terminal tetrasaccharide characterized by the addition of sialic acids in a α 2,3 linkage to the galactose residue in type II structures. This addition is mainly performed by the members of the α 2,3 ST subfamily, ST3Gal III, IV and VI [14].

SLeX is normally expressed in immune and inflammatory cells upon cytokine stimuli, triggered during inflammation, and is recognized as a selectin binding/ligand to endothelial cells. This sweet harmony leads to immune cell adhesion to endothelia and further facilitates cell rolling and extravasation to the inflammatory site [15]. In such a way, cancer cells that express SLeX take advantage of this process to easily travel through the bloodstream and form secondary metastasis in distant organ sites [11].

Previously, using a ST3GalIV overexpression cellular model, we reported the role of ST3GalIV in SLeX expression in gastric cancer cells [16, 17] and its association with the activation of c-Met signaling pathway [18]. More recently, we showed that SLeX is expressed in aggressive gastric carcinomas, highlighting its clinical value as a prognostic biomarker and an appealing therapeutic target [12]. These results compelled us to deeply explore the role of SLeX in cancer. In this study, we further elucidate the role of ST3GalIV in SLeX expression and the malignant properties of GI cancer cells, through the establishment of glycoengineered cellular KO models to comprehensively understand the specific contribution of distinct ST3Gals.

Materials and methods

Cell lines and cell culture

KatoIII, NCI-N87, SNU1, SNU638, Colo205, LS174T, HT-29, HCT15, HCT116, RKO, DLD1, SW48, SW480, SW620 and Caco-2 were obtained from American Type Culture Collection (ATCC). The cells were grown in monolayer culture in 6-well plates (Fisher Scientific) and maintained at 37 °C in an atmosphere of 5% CO₂, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX, HEPES medium, Dulbecco's Modified Eagle Medium (DMEM) or Iscove

Modified Dulbecco Media (IMDM) (all from Gibco), all supplemented with 10% fetal bovine serum (FBS) (Biowest).

RNA extraction and reverse transcription PCR

Total RNA was extracted from cellular pellets using the TRI Reagent (Sigma) protocol, according to manufacturer instructions. Isolated RNA was then quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and converted to cDNA by reverse transcription (RT). RT was performed using a mixture containing 3 μ g of total RNA, 1 μ L of random oligonucleotides primers, 1 μ L of 10 mM deoxynucleotide triphosphates (dNTPs), 4 μ L of 5X RT buffer, 1 μ L of 100 mM dithiothreitol (DTT), 1 μ L of RNaseOUT (40 U/ μ L), 1 μ L of SuperScript IV reverse transcriptase (200 U/ μ L) (Invitrogen) and nuclease-free water in a final reaction volume of 20 μ L, according to the manufacturer's protocol. Briefly, RT reactions involve three main steps: primer annealing (65 °C, 5 min; 4 °C, > 1 min), DNA polymerization (23 °C, 10 min; 50–55 °C, 20 min) and enzyme deactivation (80 °C, 10 min). A mixture without RNA was used as control for RT.

Real-time PCR (q-PCR) analysis

For real-time PCR analysis, 2 μ L of cDNA samples (diluted 30-fold with Nuclease-Free Water), 0.6 μ L of each 10 mM primer and 10.0 μ L Power SYBRGreen Master Mix (Applied Biosystems) were amplified using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used are summarized in Table 1. Expression analysis of β -Actin housekeeping gene was also measured in triplicate for each sample and used for normalization of target gene abundance. Specificity of amplification was confirmed by melting curve analysis. Standard curves were determined for each gene, and results are presented as ratio between target gene and the housekeeping gene, β -Actin.

CRISPR/Cas9 knockout

ST3GAL4 and ST3GAL6 knockout were performed using CRISPR/Cas9 technology as described previously [19]. Briefly, cells were co-transfected with a Cas9

Table 1 Primer sequences for qRT-PCR analysis of gene expression

Gene	Primer Forward	Primer Reverse
ST3GAL3	GGTGGCAGTCGCAGGATT	CATGCGAACGGTCTCATAGTAGTG
ST3GAL4	TGAGGTGGCCCGAGG	CCGGGAGTAGTTGCCAAA
ST3GAL6	CGGCTGATTTAGAAAGATTGCTT	CGGCTGATTTAGAAAGATTGCTT
β -Actin	AGAAAATCTGGCACCACACC	TAGCACAGCCTGGATAGCAA

endonuclease vector containing GFP and a plasmid with a validated guide RNA for the target genes (*ST3GAL4* “TTACCCGCTTCTTATCACTC” and *ST3GAL6* “TAA TACGACAGTGATTCTCA”). KO clones were obtained by fluorescence-activated single-cell sorting (FACS) for the enrichment of nuclease-expressing cells, and gene KO was validated by indel detection using amplicon and restriction fragment length polymorphism (RFLP) combination analysis, as previously described [20]. Three clones were selected for each target gene and gene KO was confirmed by Sanger sequencing. The results were analyzed through Tracking of Indels by Decomposition (TIDE) methodology [21].

Immunofluorescence

All cell lines and respective KO cell models were seeded in 12-well chamber slides (ibidi) or in 13 mm coverslips (Marienfeld). The cells were washed with phosphate-buffered saline (PBS) and fixed with methanol during 20 min at -20 °C. Blocking of immunoglobulins cross-reaction was performed using goat serum (1:5) (Dako) in 10% BSA in PBS at RT for 30 min, followed by primary antibody incubation at 4 °C overnight. Slides were then incubated with fluorescently-labeled secondary antibodies anti-mouse IgM Alexa Fluor® 594 (1:500) or IgG Alexa Fluor® 488 (1:750) (Thermo Fisher Scientific) for 1 h at RT. Nuclear counterstaining was performed by incubating cells with 4',6'-diamino-2-phenyl-indol (DAPI) for 10 min and slides or coverslips were mounted with VectaShield (Vector Laboratories). The fluorescent signal was examined using a fluorescence microscope and images were acquired with Zeiss Axio Imager Z1, Zeiss AxioCam MR version 3.0, and the AxioVisionRel (version 4.8) software (Carl Zeiss). Images were acquired under 200x and 400x (Fig. 5 and S3) magnifications.

The primary antibodies used were: SLeX (CSLEX, 1:250, BD Bioscience), SLeA (CA19-9, 1:200, Abcam), LeX (SH1, 1:5, [22]) and LeA (CA3F4 1:5, [23]).

Flow cytometry analysis

Cells previously cultured in 25cm² flasks were detached with accutase (Gibco), resuspended in fresh medium and centrifuged at 300 g for 5 min. Cells were adjusted to 1 × 10⁶ cells/ml and then washed two times in PBS with 1% bovine serum albumin (BSA, Sigma–Aldrich) solution. The cell pellets were resuspended and incubated 30 min at 4 °C with the primary antibodies for SLeX, SLeA, LeA and LeX at the same dilutions used for immunofluorescence assay. After

two washes, cells were incubated with AlexaFluor647 fluorochrome-conjugate antimouse IgM for SLeX detection and with AlexaFluor488 fluorochrome-conjugate antimouse IgG for SLeA, LeA and LeX, during 30 min at 4 °C. Negative controls were performed by using cells stained only with secondary antibodies. Cells were washed, resuspended in PBS 1% BSA solution and filtered prior analysis. Data acquisition was performed using BD LSRFortessa and analyzed with the FlowJo software (v10; BD Biosciences). Three independent experiments were analyzed.

SDS-PAGE and western blot analysis

Whole cell lysates were obtained from subconfluent cell cultures using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined by the bicinchoninic acid protein assay (BCA) (Pierce), and 30 µg of protein extracts were loaded onto 7.5% sodium dodecyl-polyacrylamide gels or 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10 or 15-well (Bio-Rad) for electrophoretic separation (SDS-PAGE) (Bio-Rad). Gels were transferred onto nitrocellulose membranes (Amersham) and blocked with 5% bovine serum albumin (BSA) (Sigma–Aldrich) in PBS containing 0.05% Tween 20 (Sigma-Aldrich) (PBS-T). Western blotting was performed by incubating the membranes with primary antibodies probed overnight (4 °C) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgM (1:15000) (Jackson ImmunoResearch) or goat anti-rabbit IgG (1:2000) (Santa Cruz) secondary antibodies. Protein bands were visualized using the ECL WB detection reagent and films (GE Healthcare).

The following primary antibodies were used: Sialyl Lewis X (SLeX, CSLEX, 1:500, BD Bioscience); β-Actin (1:2000, CellSignaling). β-Actin was used as loading control.

Cell proliferation assay

Cell proliferation/viability was analyzed *in vitro* by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using a commercially available kit (MTT Cell Proliferation Kit I, Roche). Cells were seeded into 96-well plates (Fisher Scientific) at the density of 1 × 10⁴ cells/well, and MTT Kit solutions added into each well according to cell proliferation kit (Roche) for each time point 24, 48 and 72 h, after seeding. The absorbance value was read at 600 nm using a microplate reader. Three independent assays were performed in triplicates and results are presented as means ± SEM for each sample, and proliferation levels obtained were normalized and compared with the wild-type (WT) cell line.

Wound healing assay

Cell motility was assessed *in vitro* by a wound healing assay. 2-well silicone ibidi inserts (ibidi) were applied to a 6-well plate (Fisher Scientific) and approximately 1×10^5 cells were seeded in each side of the 2-well and left to adhere for 24 h. The insert was then carefully removed, leaving a defined gap area (wound) between the cells. After being washed twice with PBS, the cells were then allowed to migrate into the cell-free area. The cells were imaged at 0, 24, 48, and 72 h at a magnification of 100x. The percentage of wound closure was calculated by measuring the free space at each time-point, normalized to the initial wound area (time point-0 h) using ImageJ software (FIJI). Three biological replicates were performed and the results are shown as average $\pm$ SEM.

Statistical analysis

Data were analyzed with GraphPad Prism 8.0 software (GraphPad Software Inc.) Quantitative data were expressed as the mean $\pm$ SEM of three biological replicates. To compare the differences between each KO cell model in regard to each respective WT values, a two-way ANOVA with a 95% interval of confidence was performed. P-values of ≤ 0.05 were considered statistically significant.

Fig. 1 Assessment of ST3GalIV and SLeX expression in a panel of gastrointestinal cancer cell lines. (a) ST3GalIV transcript expression levels showing an heterogeneous expression across the distinct cancer cell lines, with DLD1, HT-29, KatoIII, LS174T, Colo205 and NCI-N87 presenting the highest levels. Bar graph shows the mean relative quantification values normalized to the internal control gene ACTB. (b) Immunofluorescence analysis of SLeX in GI cell lines showing positive staining in HT-29, KatoIII, LS174T, Colo205 and NCI-N87 cell lines. (c) SLeX-protein expression by western blot analysis showing a wide range of SLeX positive glycoproteins in HT-29, KatoIII, LS174T, Colo205 and NCI-N87 cells. The scale bar corresponds to 100 μ m

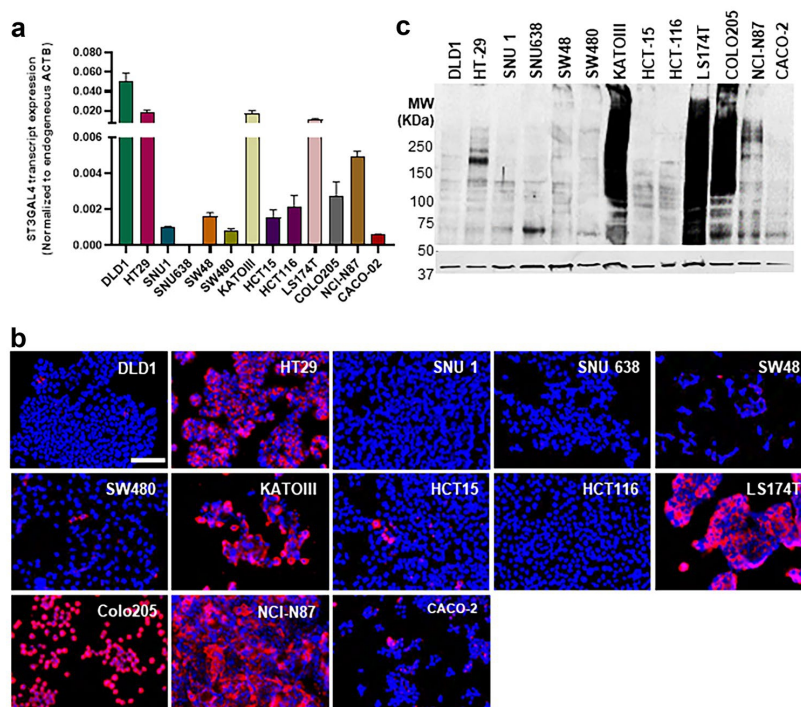
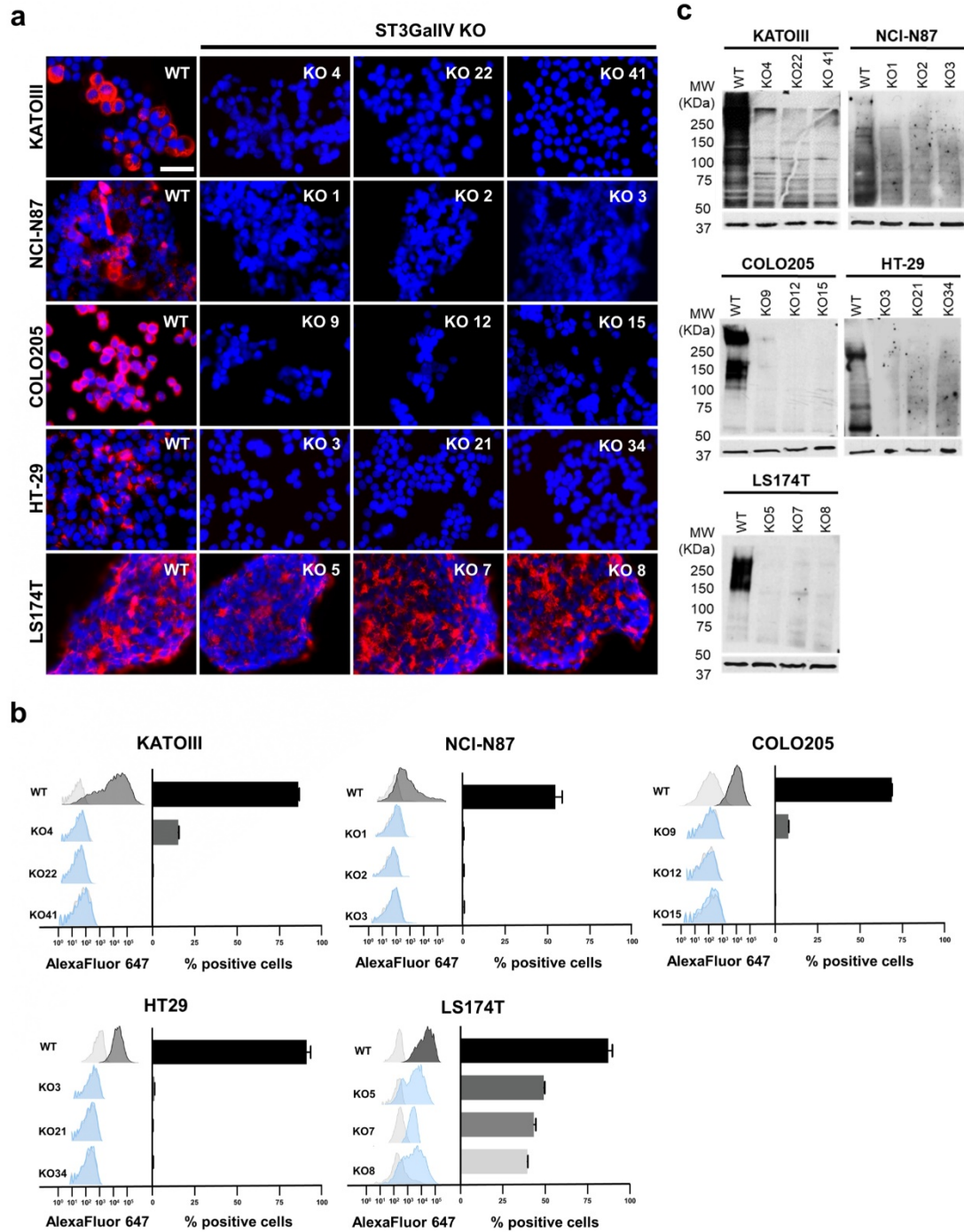


Fig. 2 ST3GalIV KO abolished SLeX expression in KatoIII, NCI-N87, Colo205, HT-29 and LS174T cell lines. Immunofluorescence (a) and flow cytometry (b) analysis showing the abolishment of SLeX in KatoIII, NCI-N87, Colo205 and HT-29 cells upon ST3GalIV KO. The exception is the colon cell line LS174T that still presenting immunodetection at the cell surface. Western blot analysis showed the complete abolishment of SLeX expression upon ST3GalIV KO in all cell lines (c). The scale bar corresponds to 50 μ m

Results

Expression of ST3GalIV and SLeX in gastrointestinal cancer cell lines

To select GI cell lines to further study the role of ST3GalIV in SLeX biosynthesis and expression and malignant characteristics of cancer cells, the transcript levels of ST3GalIV were initially assessed in a panel of 13 GI cancer cell lines. ST3GalIV shows a heterogeneous expression within the studied cell lines (Fig. 1a), with DLD1, HT-29, KatoIII, LS174T, Colo205, and NCI-N87 presenting the highest enzyme levels. Furthermore, we analyzed SLeX expression at the cell surface (Fig. 1b) and specifically at the protein level (Fig. 1c) and identified Kato-III and NCI-N87 (gastric cancer cells) and HT-29, LS174T and Colo205 (colorectal cancer cells) as SLeX positive. Thus, these five SLeX positive cell lines were selected for ST3GalIV gene KO.



ST3GalIV gene KO impairs SLeX expression mainly at the protein level

ST3GalIV KO was performed in the selected cell lines, as previously described using a validated gRNA for *ST3GAL4* [19], and three independent isogenic cell clones were selected for further experiments (Table 2 and Fig. S1). Immunofluorescence analysis for SLeX showed the complete abrogation of SLeX in almost every cell line. The only exception was the colorectal cancer cell line LS174T (Figs. 2a and b and S2). On the other hand, protein analysis by western blot showed the complete abolishment of SLeX expression in all cell lines upon ST3GalIV KO (Fig. 2c). This result pointed to a role of another sialyltransferase in SLeX expression in LS174T cells, likely at the lipid level.

In addition, we evaluated the impact of ST3GalIV KO in the biosynthesis of the SLeX isomer SLeA and in the non-sialylated Lewis antigens LeA and LeX. Flow cytometry and immunofluorescence analysis in WT cells showed a heterogeneous expression of SLeA (Figs. 3 and S3, left panel for each cell line). Colo205 cells presented SLeA positive expression in almost all cells, KatoIII cells presented a high expression with around 80% positive cells and HT-29 cells, NCI-N87 and LS174T cells showed few positive cells (Figs. 3 and S4). Interestingly, ST3GalIV KO led to a reduced SLeA detection

in all positive cell lines. Regarding the expression of the non sialylated Lewis antigens LeA and LeX, ST3GalIV KO lead to an increased detection and/or increased number of positive LeA and LeX cells when compared to parental cells (Figs. 3 and S3 middle and right panel for each cell line and Fig. S4). The only exception was the expression of LeA in KatoIII ST3GalIV KO cells that was diminished. Regarding LS174T cells, no no major differences were observed in ST3GalIV KO cells when compared to parental cells for both LeA and LeX detected intensity or number of positive cells (Figs. 3, S3 and S4).

ST3GalIV-driven SLeX expression impacts the motility of cancer cells

To infer the role of ST3GalIV-associated SLeX expression in the malignant properties of cancer cells, we evaluated classical biological parameters such as cell proliferation and cell motility. Results showed that ST3GalIV has a significant impact on the motility capacity of cells, as can be appreciated by the reduced capacity of ST3GalIV KO cells to close the wound along 72 h (Fig. 4b, d, f, and h). The only exception was the LS174T ST3GalIV KO cells, where no differences were observed in cell motility when compared to WT cells (Fig. 4j). Regarding cell proliferation, both NCI-N87 and LS174T ST3GalIV KO cells presented a significant reduction in the proliferation rate assessed at selected time-points, when compared to the parental cell line (Fig. 4c and i). The other KO cell lines presented punctual statistically significant differences (Fig. 4a and e) or no effect (Fig. 4g) when compared to WT parental cells.

ST3GalVI is also a major contributor for SLeX biosynthesis in LS174T cells and promotes cellular motility

Since LS174T cells retain SLeX expression after ST3GalIV KO, likely at the lipid level, we sought to investigate the role of other ST3Gals in SLeX biosynthesis in this cell line. We analyzed the expression levels of ST3GalIII (Fig. 5a) and ST3GalVI (Fig. 5b) in the studied cell lines and observed that LS174T expresses both enzymes with significantly levels for ST3GalVI. Since ST3GalVI role in SLeX biosynthesis has been robustly established in previous studies [13, 24, 25], particularly in glycolipids [26], we decided to perform a ST3GalVI KO in LS174T WT cells and in the established LS174T ST3GalVI KO clones. We observed that ST3GalVI KO led to a significant reduction in SLeX in LS174T WT cells (Fig. 5c middle panel and d). In addition, at the protein level we observed that ST3GalVI KO led to the abolishment of SLeX expression (Fig. 5e). The ST3GalVI KO did not have any impact in neither cell proliferation (Fig. 5f) nor

Table 2 Summary of ST3GalVI and ST3GalIV indels present in the different gastrointestinal 2 KO cell lines

CELL LINES		ST3GALIV KO	ST3GALIV KO
COLO205	Clone 9	-4/-1	-
	Clone 12	-1/+1	-
	Clone 15	-1	-
HT-29	Clone 3	-1	-
	Clone 21	-2/-1	-
	Clone 34	-5/-2	-
LS174T	Clone 5	+1	-
	Clone 7	-1/+1	-
	Clone 8	-2	-
NCI-N87	Clone 1	-4/-2	-
	Clone 2	-2	-
	Clone 3	-2/-1	-
KATOIII	Clone 4	-2/-1/+1	-
	Clone 22	-4/-2/-1	-
	Clone 41	-8/-5/-4/-2/+1	-
LS174T	Clone 4	-	-4/-1
	Clone 15	-	-17/-9
	Clone 17	-	+1
LS174T	Clone 5	+1	-1
	Clone 4	-1/+1	-5/-1
	Clone 2	-2	+1

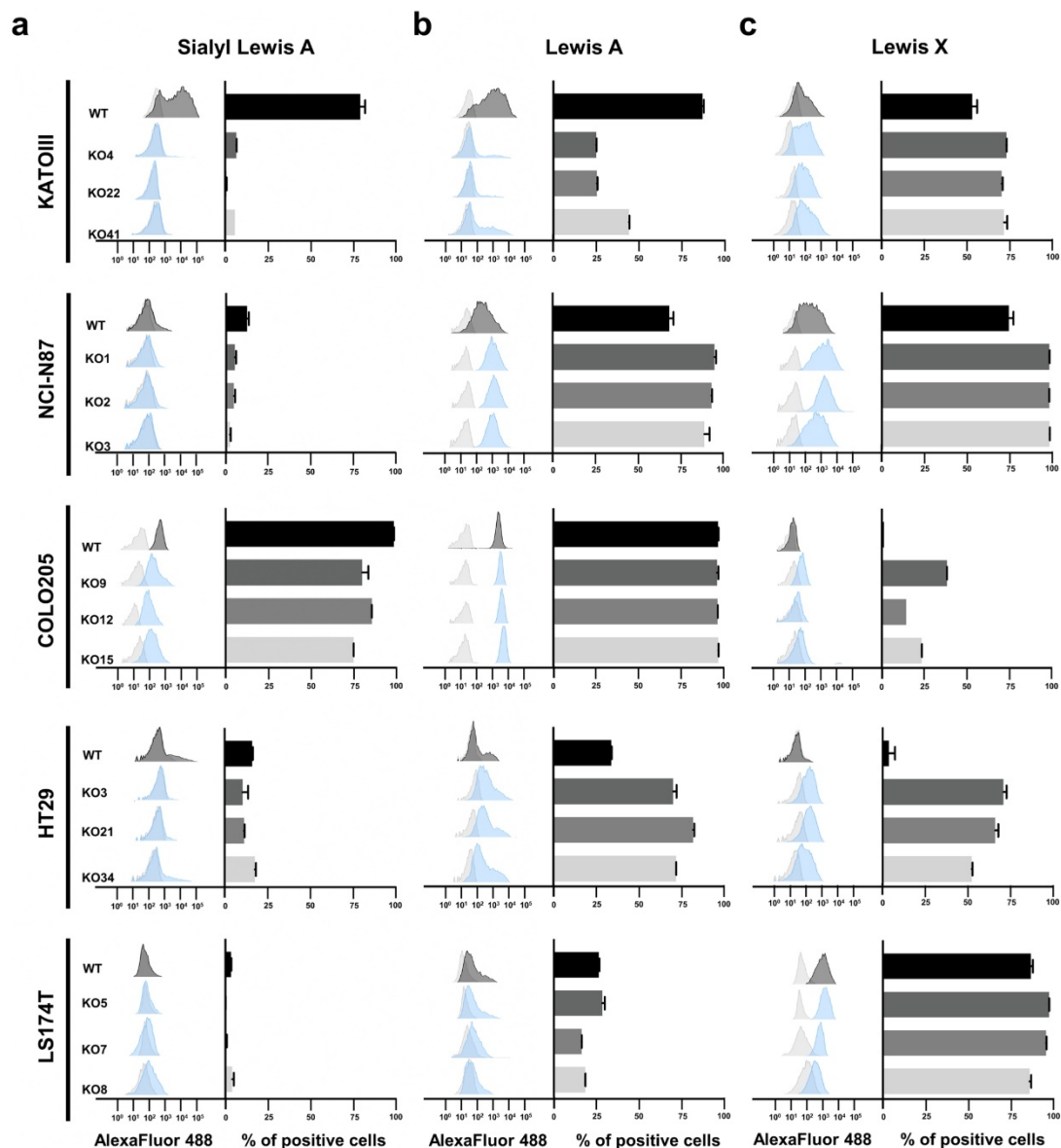


Fig. 3 Flow cytometry analysis of SLeA, LeA and LeX in ST3GalIV KO GI cancer cells and parental cells. Levels (histograms) and percentage of positives cells (bar graphs) of cell surface detected SLeA (a), LeA (b) and LeX (c) in KATOIII, NCI-N87, COLO205, HT-29, LS174T. Results showing a heterogenous expression of SLeA, LeA

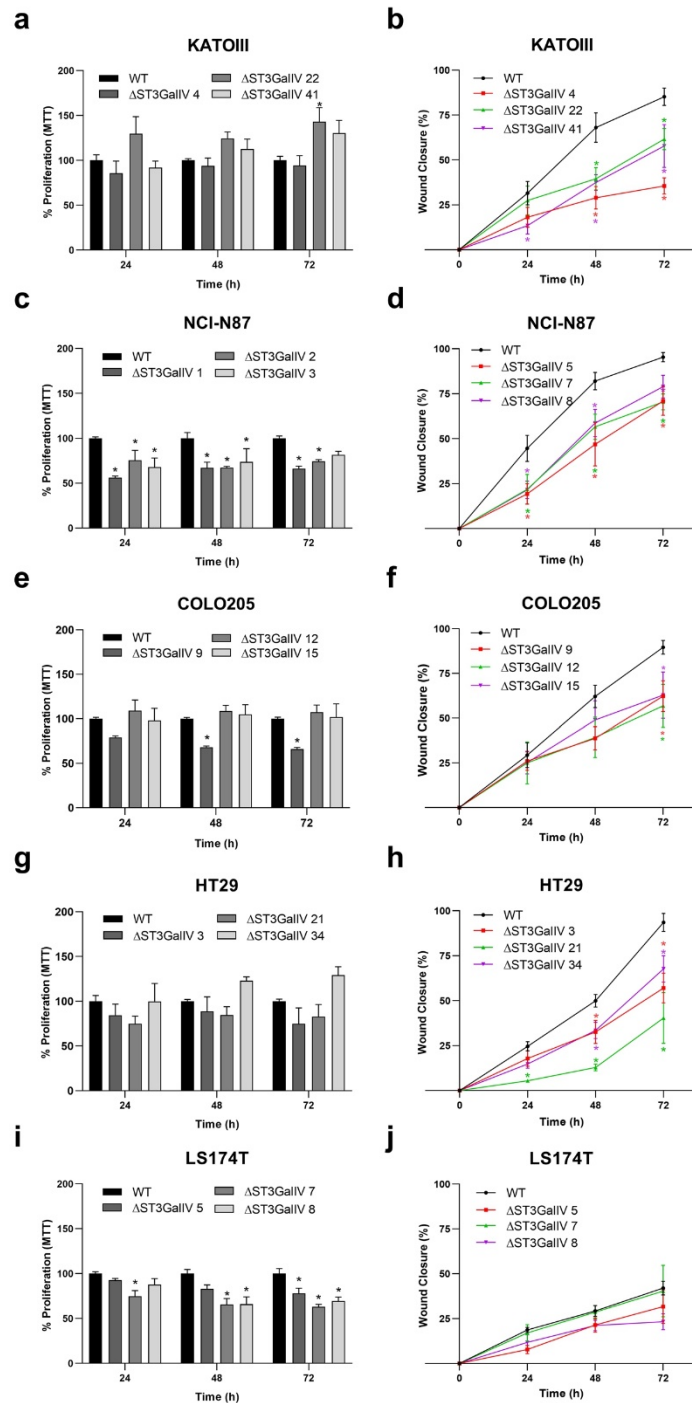
and LeX in the selected WT GI cancer cells. Overall, ST3GalIV KO cells present reduced levels of SLeA detection in positive cells, and an increased detection of the LeA and/or LeX. The only exception is the colon cell line LS174T. Light gray shaded peaks indicate the secondary control

motility capacity (Fig. 5g). On the other hand, the ST3GalVI KO in LS174T ST3GalIV KO clones revealed a complete abolishment in SLeX expression (Fig. 5c down panel and d), with a significant impact in the cellular motility (Fig. 5i), but not in the cellular proliferation (Fig. 5h).

Discussion

SLeX expression is an indisputable feature of immune response activation [27], in addition to playing a pivotal role in leukocyte adhesion and rolling through the endothelium

Fig. 4 Biological impact of ST3GalIV in the selected GI cancer cell lines. (a), (e), (g), (i), showing no differences in cell proliferation upon ST3GalIV KO, with the exception of the gastric cancer cells NCI-N87. (c), (b), (d), (f), (h) Showing the statistically significant reduction in cell motility upon ST3GalIV KO with the exception of LS174T KO cells (j). * $p \leq 0.05$



during inflammation [15]. Beyond that, SLeX is increasingly expressed in many carcinomas being associated with motility and invasive properties of cancer cells [8, 11, 18, 28, 29]. Structurally, SLeX is a terminal tetrasaccharide attached to proteins and/or lipids present at the cell surfaces or secreted ones. This tetrasaccharide is composed of a fucose α 1,3 linked to N-Acetylglucosamine and a sialic acid α 2,3 linked to a galactose, both in type II LacNAc chains [30]. The terminal addition of sialic acid in SLeX glycans is described to be performed by members of the ST3Gal family, specifically by ST3GalIII, ST3GalIV, and ST3GalVI. However, it is known that ST3Gal III preferentially acts on type I glycan chains giving rise to the SLeX isomer SLeA, with very low catalytic efficiency towards type II glycan chains [31]. Nevertheless, studies have demonstrated the role of ST3GalIII in SLeX expression for instance, in cancer [32, 33] and in egg implantation [34].

In this study, we wanted to disclose the role of ST3GalIV in SLeX expression and malignant properties of GI cancer cell lines. We prioritized GI cancers as *in vitro* models of disease due to the documented high SLeX expression in these tumors [11]. Additionally, since these cancers are listed on the very top of rankings regarding prevalence and mortality [35], understanding the way SLeX is expressed and impact cancer cell associated biological properties is extremely valuable in the quest for novel therapeutic targets. Thus, we took advantage of an available library of validated gRNAs [19] to specifically abolished the ST3GalIV enzyme expression in SLeX-positive cancer cell lines and observed a complete absence of SLeX expression in the selected clones. In addition, we observed a reduction in SLeA and an overall increased expression of the non sialylated Lewis X and A. The only exception in this study was the LS174T colon cancer cell line, which still retained SLeX expression at the cell surface, only detected by immunofluorescence, suggesting the presence of SLeX-modified glycolipids. In addition, LS174T cells were completely negative for SLeA in both WT and ST3GalIV KO cells. In the late twentieth century, the *in vitro* preference of this enzyme towards type I and II glycan chains, when using ST3GalIV recombinant enzyme, was described and further corroborates our results [36]. Using a soluble form of ST3GalIV from the human placenta, Kitagawa *et al.* also showed the catalytic capacity of this enzyme on both glycoproteins and glycolipids containing type II chains or Gal β 1-3GalNAc structures, but not on type I chains [37]. In addition, TNF-induced ST3GalIV expression with consequent SLeX upregulation has been described in lung epithelial cells and human bronchial mucosa [38–40]. Herein, our results, using 4 different ST3GalIV KO GI cancer cell lines, showed the lack of type II sialylation, through the abolishment of SLeX detection, and a reduction in type I sialylation, through the reduction in SLeA detection upon ST3GalIV KO. Thus, our results

demonstrate ST3GalIV catalytic capacity on both type I and type II chains. In the literature, it has been shown that ST3GalIII is the major player in SLeA biosynthesis. However, Indelicato and collaborators recently described a nonsense inactivating ST3GAL3 mutation in two patients with congenital disorder of glycosylation in which, contrary to what was expected, it still abundantly expressed circulating SLeA [41]. The authors hypothesized that ST3GAL3 deficiency may partially be compensated by other members of the ST3Gal family and that gangliosides may also play a role. In our work we showed that ST3GalIV KO not only clearly impact the biosynthesis of SLeX but also SLeA. This finding corroborates the above-mentioned study that showed that ST3GAL3 deficiency was not sufficient to impair SLeA biosynthesis, supporting a compensatory coordinated action of STs enzymes.

Further, in colon LS174T cells a likely glycolipid-associated SLeX expression was still observed in ST3GalIV KO cells. Indeed, the major contribution of gangliosides as SLeX/A main carriers has been already characterized by mass spec analysis in WT LS174T cells [42]. Thus, since ST3GalVI was described as acting on type II glycan chains, mostly on glycoproteins and glycolipids [26], we decided to perform ST3GalVI KO in both WT and ST3GalIV KO LS174T. Indeed, ST3GalVI KO on WT cells led to a reduced expression of SLeX expression at the cell surface and its abolishment at the protein level. Furthermore, the double KO led to the complete abrogation of SLeX expression. Nonetheless, the fact that either ST3GalIV or ST3GalVI KO alone led to the complete abolishment of SLeX at the protein level in this cell line is quite puzzling, showing STs redundancy. Regarding this result, we can speculate about an enzymatic complex comprising both enzymes, essential for protein Lewis X sialylation in LS174T cells.

SLeX expression in cancer has been associated with patient's invasive tumors and dismal prognosis [12, 43], and for this reason, one of our objectives was to evaluate the biological impact of inhibiting SLeX biosynthesis in selected GI cancer cells. We observed that SLeX inhibition did not affect cancer cell proliferation overall. Only NCI-N87 cells showed statistically significant reduction in all the analyzed time-points for the three isogenic clones. The remaining cell lines showed punctual statistically significant differences, that might suggest a clonal selection effect rather than ST3GalIV KO biological effect. However, a complete SLeX inhibition led to a reduced motility capacity in all studied cell lines. This effect was observed in the ST3GalIV KO cells NCI-N87, KatoIII, Colo205 and HT-29 and in the double ST3GalIV/VI KO in LS174T cells. This result corroborates our previous findings that showed an increased invasive capacity of gastric cancer cells when we induced SLeX expression through ST3GalIV overexpression [18]. In addition, other studies that showed the importance of SLeX

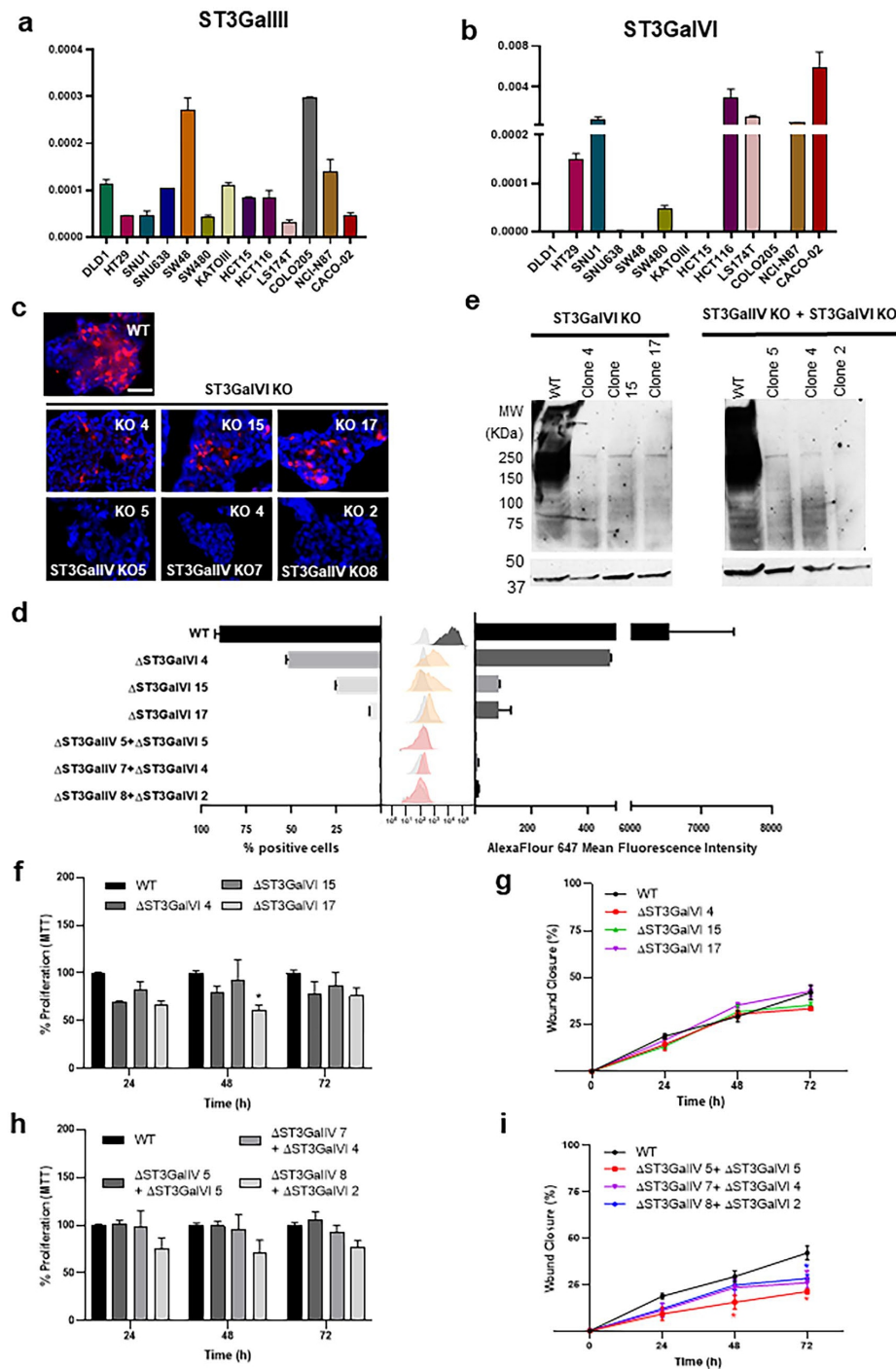


Fig. 5 Role of ST3GalVI in SLeX expression and malignant properties of LS174T cells. **(a)** Expression analysis of ST3GalIII in a panel of GI cancer cell lines showing a heterogeneous expression within cell lines, with LS174T presenting the lowest expression. **(b)** Expression analysis of ST3GalVI in a panel of GI cancer cell lines showing a heterogeneous expression within cell lines, with LS174T presenting a significant expression. Bar graph shows the mean relative quantification values for each gene, normalized to the internal control gene ACTB. **(c)** immunofluorescence analysis of LS174T ST3GalVI KO cells showing a reduced SLeX expression in ST3GalVI KO clones and a complete abolishment of expression in ST3GalIV/VI KO clones. The scale bar corresponds to 50 μ m. **(d)** Expression levels and percentage of SLeX positive cells measured by flow cytometry analysis, showing a complete abolishment of SLeX detection in the double KO cells. **(e)** Western blot analysis of ST3GalVI KO clones showing the capacity of ST3GalVI and ST3GalIV/VI KO in impair SLeX expression at the protein level. **(f), (h)** Proliferation analysis in ST3GalVI KO clones showing no statistically significant differences. **(g), (i)** Motility capacity of ST3GalVI KO clones showing a reduced cell motility only in ST3GalIV/VI KO clones

induction through cytokine stimuli, showed its role in cancer cell motility and invasion [28].

Overall, our findings demonstrate the role of ST3GalIV in the biosynthesis of SLeX in GI cancer cells, and its association with the cellular motility. Moreover, we showed that, in particular cases (e.g. LS174T cells), ST3GalIV is not solely responsible in ensuring SLeX biosynthesis.

Abbreviations SLeX: Sialyl Lewis X; SLeA: Sialyl Lewis A; LeA: Lewis A; LeX: Lewis X; α 2: β -Galactoside; ST3Gals: 3-Sialyltransferases; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; KO: Knock-Out; GI: Gastrointestinal

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10719-023-10113-y>.

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Authors Contributions CG and CAR supervised the study. CG designed the experiments. CG establish the KO models. AFC, ES, IFR, AT, JM, MP and CG performed experiments and analyzed the data. CG wrote the original paper. All authors critically revised the paper.

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Data availability The cellular models established during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Consent for publication The authors listed in this manuscript consented to publish this manuscript.

Competing interests The authors declare that they have no competing interests.

Ethical approval This article does not contain any data derived from studies with human participants or animals.

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References

- Ohtsubo, K., Marth, J.D.: Glycosylation in cellular mechanisms of health and disease. *Cell* **126**(5), 855–867 (2006)
- Mercier, S., Balmana, M., Campos, D., Gomes, J., Reis, C.A.: Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? *Cancer Cell* **36**(1), 6–16 (2019)
- Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Mohnen, D., Kinoshita, T., Packer, N.H., Prestegard, J.H., Schnaar R.L., Seeberger, P.H.: *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor Laboratory Press (2022)
- Gu, J., Isaji, T., Xu, Q., Kariya, Y., Gu, W., Fukuda, T., Du, Y.: Potential roles of N-glycosylation in cell adhesion. *Glycoconj J* **29**(8–9), 599–607 (2012)
- Zhang, H., Freitas, D., Kim, H.S., Fabijanic, K., Li, Z., Chen, H., Mark, M.T., Molina, H., Martin, A.B., Bojmar, L., Fang, J., Rampersaud, S., Hoshino, A., Matei, I., Kenific, C.M., Nakajima, M., Mutvei, A.P., Sansone, P., Buehring, W., Wang, H., Jimenez, J.P., Cohen-Gould, L., Paknejad, N., Brendel, M., Manova-Todorova, K., Magalhaes, A., Ferreira, J.A., Osorio, H., Silva, A.M., Massey, A., Cubillos-Ruiz, J.R., Galletti, G., Giannakou, P., Cuervo, A.M., Blenis, J., Schwartz, R., Brady, M.S., Peinado, H., Bromberg, J., Matsui, H., Reis, C.A., Lyden, D.: Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol* **20**(3), 332–343 (2018)
- Zabczynska, M., Polak, K., Kozłowska, K., Sokolowski, G., Poheć, E.: The Contribution of IgG Glycosylation to Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) in Hashimoto's Thyroiditis: An *in Vitro* Model of Thyroid Autoimmunity. *Biomolecules* **10**(2) (2020)
- Cobb, B.A.: The history of IgG glycosylation and where we are now. *Glycobiology* **30**(4), 202–213 (2020)

8. Rodrigues, J.G., Balmana, M., Macedo, J.A., Pocas, J., Fernandes, A., de-Freitas-Junior, J.C.M., Pinho, S.S., Gomes, J., Magalhaes, A., Gomes, C., Mereiter, S., Reis, C.A.: Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis. *Cell Immunol* **333**, 46–57 (2018)
9. Pinho, S.S., Reis, C.A.: Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* **15**(9), 540–555 (2015)
10. Ugorski, M., Laskowska, A.: Sialyl Lewis(a): a tumor-associated carbohydrate antigen involved in adhesion and metastatic potential of cancer cells. *Acta Biochim Pol* **49**(2), 303–311 (2002)
11. Trinchera, M., Aronica, A., Dall'Olio, F.: Selectin Ligands Sialyl-Lewis a and Sialyl-Lewis x in Gastrointestinal Cancers. *Biology (Basel)* **6**(1), 66 (2017)
12. Gomes, C., Almeida, A., Barreira, A., Calheiros, J., Pinto, F., Abrantes, R., Costa, A., Polonia, A., Campos, D., Osorio, H., Sousa, H., Pinto-de-Sousa, J., Kolarich, D., Reis, C.A.: Carcinoembryonic antigen carrying SLe(X) as a new biomarker of more aggressive gastric carcinomas. *Theranostics* **9**(24), 7431–7446 (2019)
13. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M.A., Samyn-Petit, B., Julien, S., Delannoy, P.: The human sialyltransferase family. *Biochimie* **83**(8), 727–737 (2001)
14. Petit, D., Teppa, E., Mir, A.M., Vicogne, D., Thisse, C., Thisse, B., Filloux, C., Harduin-Lepers, A.: Integrative view of alpha2,3-sialyltransferases (ST3Gal) molecular and functional evolution in deuterostomes: significance of lineage-specific losses. *Mol Biol Evol* **32**(4), 906–927 (2015)
15. Somers, W.S., Tang, J., Shaw, G.D., Camphausen, R.T.: Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. *Cell* **103**(3), 467–479 (2000)
16. Mereiter, S., Magalhaes, A., Adamczyk, B., Jin, C., Almeida, A., Drici, L., Ibanez-Vea, M., Larsen, M.R., Kolarich, D., Karlsson, N.G., Reis, C.A.: Glycomic and sialoproteomic data of gastric carcinoma cells overexpressing ST3GAL4. *Data Brief* **7**, 814–833 (2016)
17. Carvalho, A.S., Harduin-Lepers, A., Magalhaes, A., Machado, E., Mendes, N., Costa, L.T., Matthiesen, R., Almeida, R., Costa, J., Reis, C.A.: Differential expression of alpha-2,3-sialyltransferases and alpha-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. *Int J Biochem Cell Biol* **42**(1), 80–89 (2010)
18. Gomes, C., Osorio, H., Pinto, M.T., Campos, D., Oliveira, M.J., Reis, C.A.: Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS ONE* **8**(6), e66737 (2013)
19. Narimatsu, Y., Joshi, H.J., Yang, Z., Gomes, C., Chen, Y.H., Lorenzetti, F.C., Furukawa, S., Schjoldager, K.T., Hansen, L., Clausen, H., Bennett, E.P., Wandall, H.H.: A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome. *Glycobiology* **28**(5), 295–305 (2018)
20. Lonowski, L.A., Narimatsu, Y., Riaz, A., Delay, C.E., Yang, Z., Niola, F., Duda, K., Ober, E.A., Clausen, H., Wandall, H.H., Hansen, S.H., Bennett, E.P., Frodin, M.: Genome editing using FACS enrichment of nuclease-expressing cells and indel detection by amplicon analysis. *Nat Protoc* **12**(3), 581–603 (2017)
21. Brinkman, E.K., Chen, T., Amendola, M., van Steensel, B.: Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* **42**(22), e168 (2014)
22. Fukushi, Y., Hakomori, S., Nudelman, E., Cochran, N.: Novel fucolipids accumulating in human adenocarcinoma. II. Selective isolation of hybridoma antibodies that differentially recognize mono-, di-, and trifucosylated type 2 chain. *J Biol Chem* **259**(7), 4681–5 (1984)
23. Young, W.W., Portoukalian, Jr., J., Hakomori, S.: Two monoclonal anticarbohydrate antibodies directed to glycosphingolipids with a lacto-N-glycosyl type II chain. *J Biol Chem* **256**(21), 10967–72 (1981)
24. Sackstein, R.: Glycosyltransferase-programmed stereosubstitution (GPS) to create HCELL: engineering a roadmap for cell migration. *Immunol Rev* **230**(1), 51–74 (2009)
25. Videira, P.A., Silva, M., Martin, K.C., Sackstein, R.: Ligation of the CD44 Glycoform HCELL on Culture-Expanded Human Monocyte-Derived Dendritic Cells Programs Transendothelial Migration. *J Immunol* **201**(3), 1030–1043 (2018)
26. Okajima, T., Fukumoto, S., Miyazaki, H., Ishida, H., Kiso, M., Furukawa, K., Urano, T., Furukawa, K.: Molecular cloning of a novel alpha2,3-sialyltransferase (ST3Gal VI) that sialylates type II lactosamine structures on glycoproteins and glycolipids. *J Biol Chem* **274**(17), 11479–11486 (1999)
27. Silva, M., Martin, K.C., Mondal, N., Sackstein, R.: sLeX Expression Delineates Distinct Functional Subsets of Human Blood Central and Effector Memory T Cells. *J Immunol* **205**(7), 1920–1932 (2020)
28. Radhakrishnan, P., Chachadi, V., Lin, M.F., Singh, R., Kannagi, R., Cheng, P.W.: TNFalpha enhances the motility and invasiveness of prostatic cancer cells by stimulating the expression of selective glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands. *Biochem Biophys Res Commun* **409**(3), 436–441 (2011)
29. Perez-Garay, M., Arteta, B., Llop, E., Cobler, L., Pages, L., Ortiz, R., Ferri, M.J., de Bolos, C., Figueras, J., de Llorens, R., Vidal-Vanaclocha, F., Peracaula, R.: alpha2,3-Sialyltransferase ST3Gal IV promotes migration and metastasis in pancreatic adenocarcinoma cells and tends to be highly expressed in pancreatic adenocarcinoma tissues. *Int J Biochem Cell Biol* **45**(8), 1748–1757 (2013)
30. Phang, R., Lin, C.H.: Synthesis of Type-I and Type-II LacNAc-Repeating Oligosaccharides as the Backbones of Tumor-Associated Lewis Antigens. *Front Immunol* **13**, 858894 (2022)
31. Kitagawa, H., Paulson, J.C.: Cloning and expression of human Gal beta 1,3(4)GlcNAc alpha 2,3-sialyltransferase. *Biochem Biophys Res Commun* **194**(1), 375–382 (1993)
32. Perez-Garay, M., Arteta, B., Pages, L., de Llorens, R., de Bolos, C., Vidal-Vanaclocha, F., Peracaula, R.: alpha2,3-sialyltransferase ST3Gal III modulates pancreatic cancer cell motility and adhesion *in vitro* and enhances its metastatic potential *in vivo*. *PLoS One* **5**(9) (2010)
33. Guerrero, P.E., Miro, L., Wong, A., Massaguer, B.S., Martinez-Bosch, N., Llorens, R., Navarro, P., Konstantopoulos, K., Llop, E., Peracaula, R.: Knockdown of alpha2,3-Sialyltransferases Impairs Pancreatic Cancer Cell Migration, Invasion and E-selectin-Dependent Adhesion. *Int J Mol Sci* **21**(17) (2020)
34. Yu, M., Wang, H., Liu, J., Qin, H., Liu, S., Yan, Q.: The sialyltransferase ST3Gal3 facilitates the receptivity of the uterine endometrium *in vitro* and *in vivo*. *FEBS Lett* **592**(22), 3696–3707 (2018)
35. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F.: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer J Clin* **0**, 1–41 (2021)
36. Sasaki, K., Watanabe, E., Kawashima, K., Sekine, S., Dohi, T., Oshima, M., Hanai, N., Nishi, T., Hasegawa, M.: Expression cloning of a novel Gal beta (1–3/1–4) GlcNAc alpha 2,3-sialyltransferase using lectin resistance selection. *J Biol Chem* **268**(30), 22782–22787 (1993)
37. Kitagawa, H., Paulson, J.C.: Cloning of a novel alpha 2,3-sialyltransferase that sialylates glycoprotein and glycolipid carbohydrate groups. *J Biol Chem* **269**(2), 1394–1401 (1994)
38. Colomb, F., Krzewinski-Recchi, M.A., Steenackers, A., Vincent, A., Harduin-Lepers, A., Delannoy, P., Groux-Degroote, S.: TNF up-regulates ST3GAL4 and sialyl-Lewisx expression in lung

- epithelial cells through an intronic ATF2-responsive element. *Biochem J* **474**(1), 65–78 (2017)
39. Colomb, F., Vidal, O., Bobowski, M., Krzewinski-Recchi, M.A., Harduin-Lepers, A., Mensier, E., Jaillard, S., Lafitte, J.J., Delannoy, P., Groux-Degroote, S.: TNF induces the expression of the sialyltransferase ST3Gal IV in human bronchial mucosa via MSK1/2 protein kinases and increases FliD/sialyl-Lewis(x)-mediated adhesion of *Pseudomonas aeruginosa*. *Biochem J* **457**(1), 79–87 (2014)
 40. Colomb, F., Krzewinski-Recchi, M.A., El Machhour, F., Mensier, E., Jaillard, S., Steenackers, A., Harduin-Lepers, A., Lafitte, J.J., Delannoy, P., Groux-Degroote, S.: TNF regulates sialyl-Lewisx and 6-sulfo-sialyl-Lewisx expression in human lung through up-regulation of ST3GAL4 transcript isoform BX. *Biochimie* **94**(9), 2045–2053 (2012)
 41. Indelicato, R., Domenighini, R., Malagolini, N., Cereda, A., Mamoli, D., Pezzani, L., Iascone, M., dall'Olio, F., Trinchera, M.: A novel nonsense and inactivating variant of ST3GAL3 in two infant siblings suffering severe epilepsy and expressing circulating CA19.9. *Glycobiology* **30**(2), 95–104 (2020)
 42. Wang, D., Madunic, K., Zhang, T., Mayboroda, O.A., Lageveen-Kammeijer, G.S.M., Wuhler, M.: High Diversity of Glycosphingolipid Glycans of Colorectal Cancer Cell Lines Reflects the Cellular Differentiation Phenotype. *Mol Cell Proteomics* **21**(6), 100239 (2022)
 43. Kolben, T., Muller, L., Meister, S., Keilmann, L., Buschmann, C., Trillsch, F., Burges, A., Czogalla, B., Mitter, S., Schmoeckel, E., Corradini, S., Mahner, S., Jeschke, U., Kessler, M., Beyer, S.: Blood group antigens SLeX, SLeA, and LeY as prognostic markers in endometrial cancer. *J Cancer Res Clin Oncol* **148**(12), 3323–3335 (2022)

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Supplementary material for:

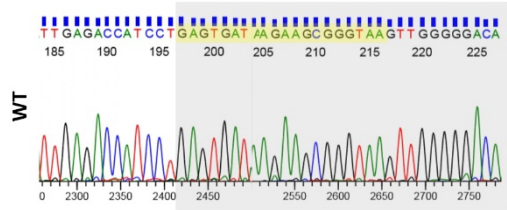
**ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and
associates with cancer cell motility**

Ana F. Costa; Emanuel Senra; Isabel Faria-Ramos; Andreia Teixeira; João Morais;
Mariana Pacheco; Celso A. Reis and Catarina Gomes

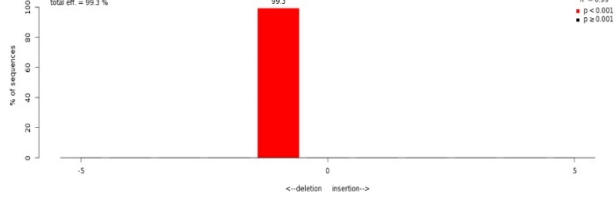
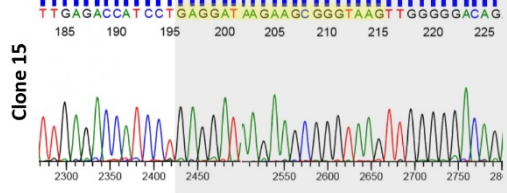
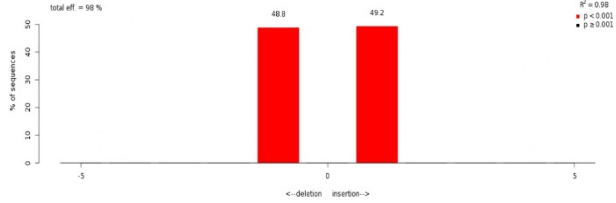
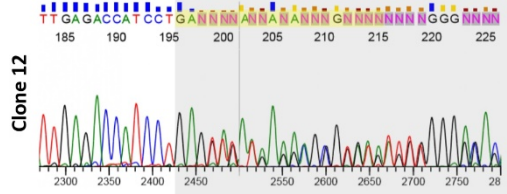
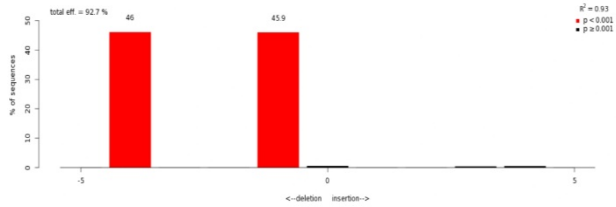
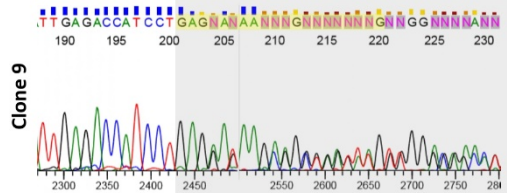
→ **Supplementary Figure 1-4**

ST3GalIV KO on COLO205, LS174T, HT-29, NCI-N87 and KatolIII gastrointestinal cancer cell lines

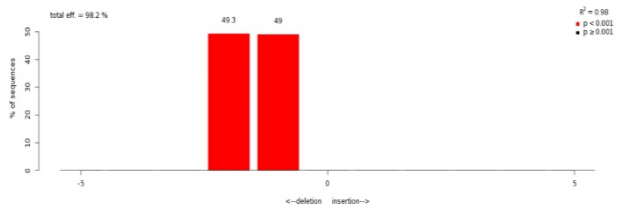
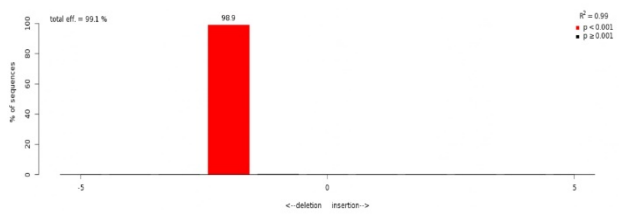
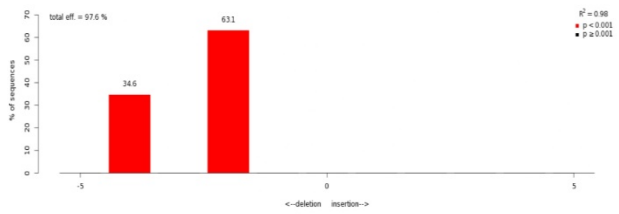
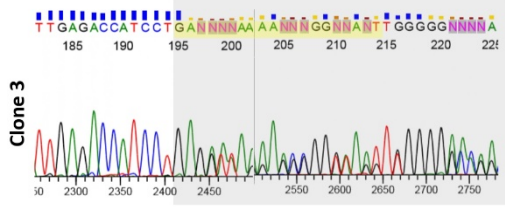
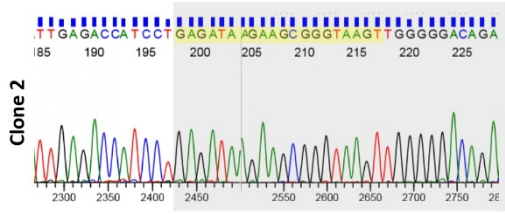
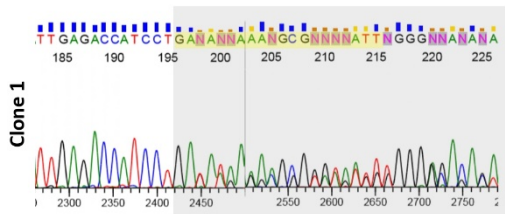
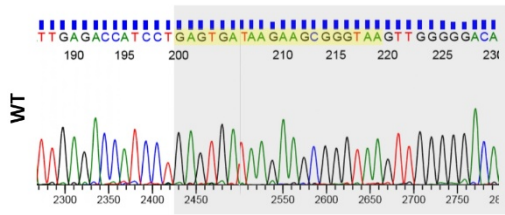
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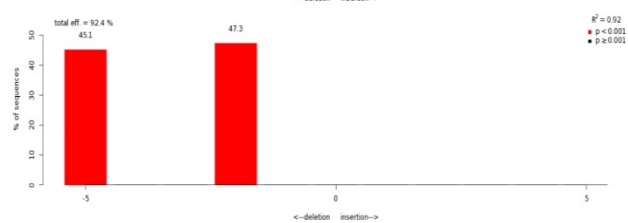
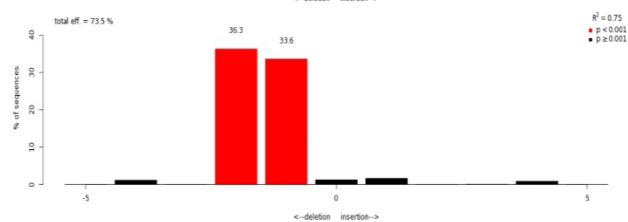
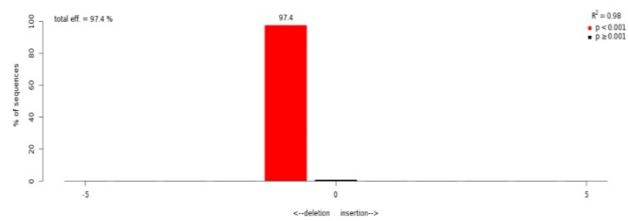
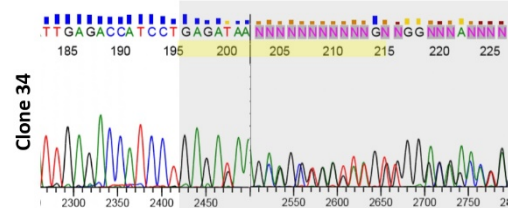
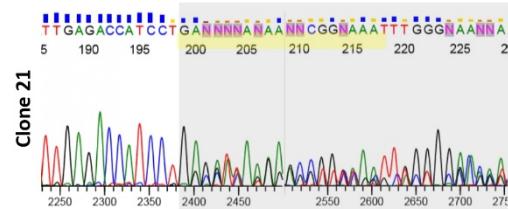
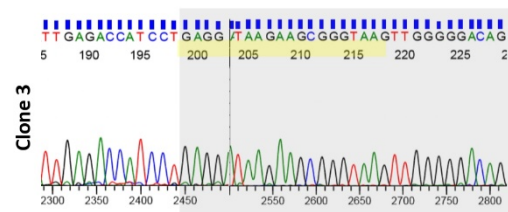
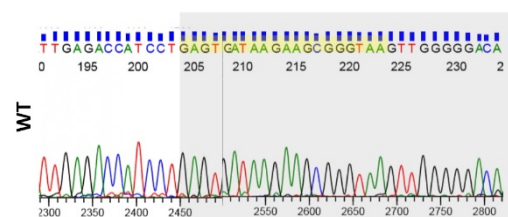


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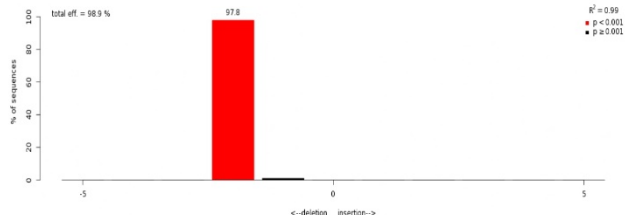
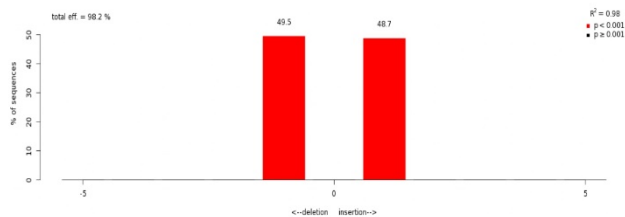
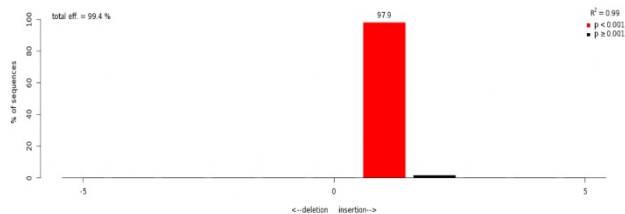
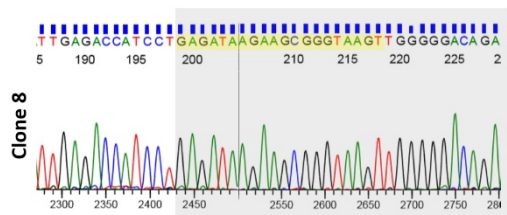
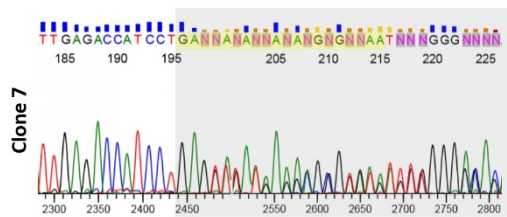
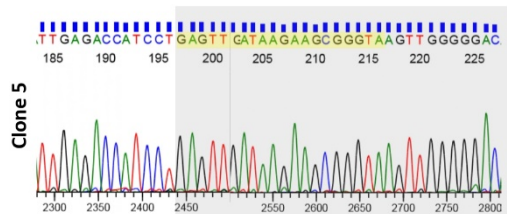
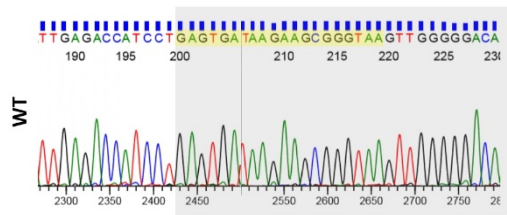


NCI-N87

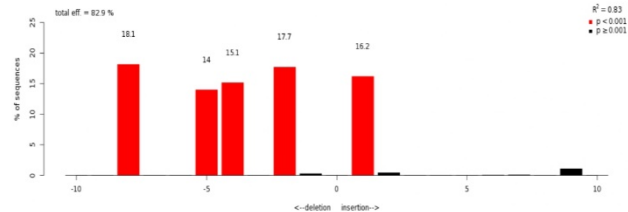
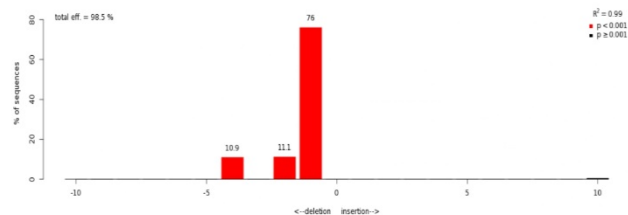
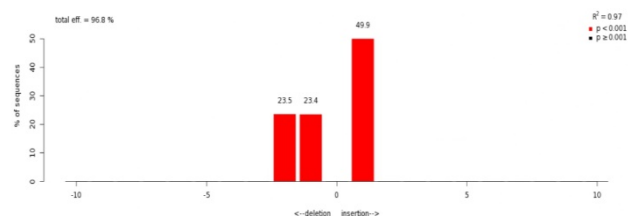
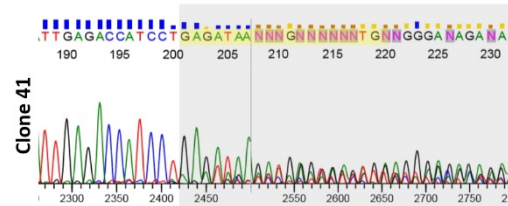
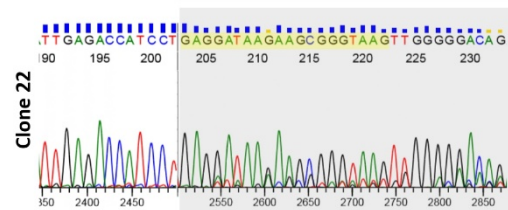
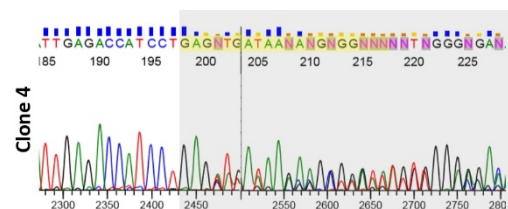
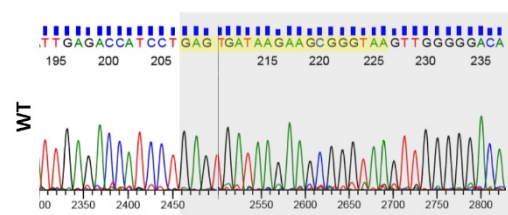


HT-29

LS174T

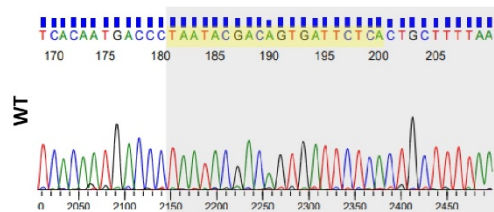


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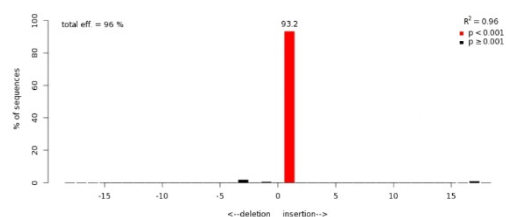
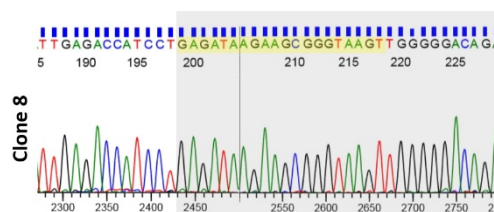
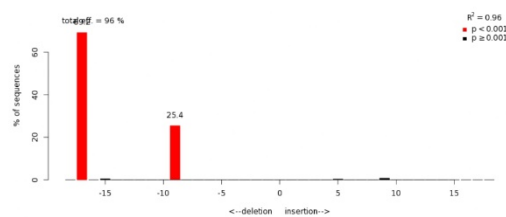
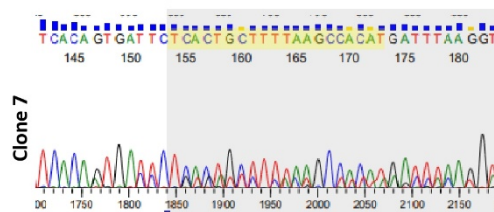
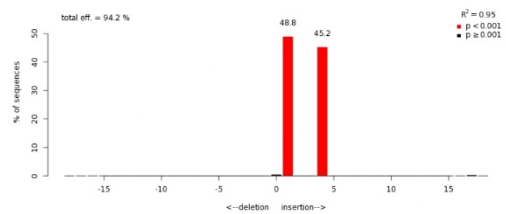
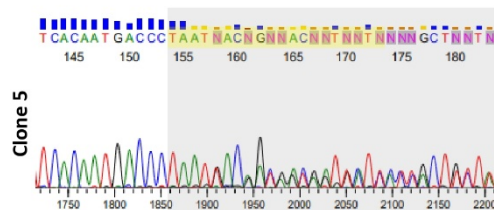
ST3GalVI KO on LS174T colon cancer cell lines

LS174T

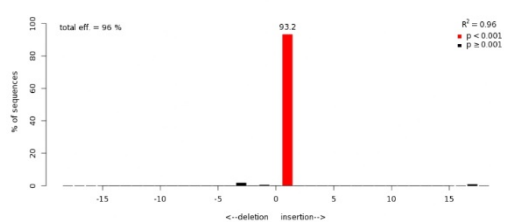
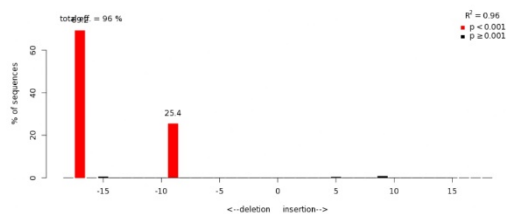
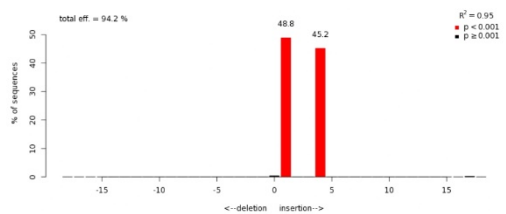
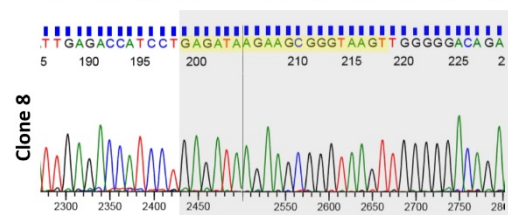
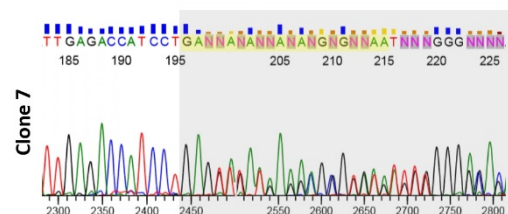
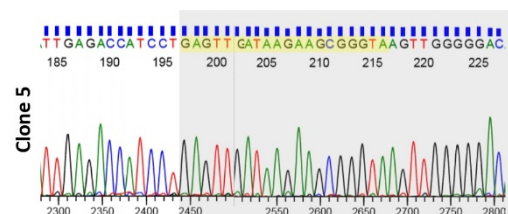
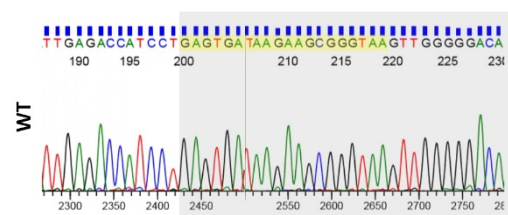


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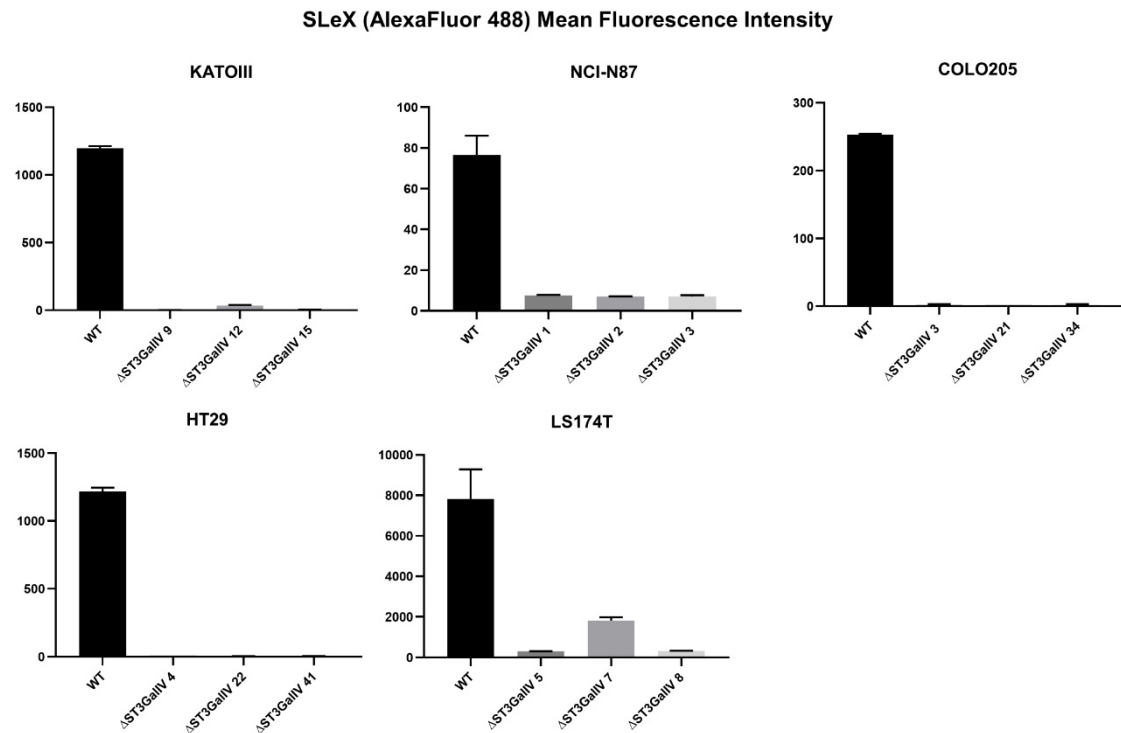
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LS174T

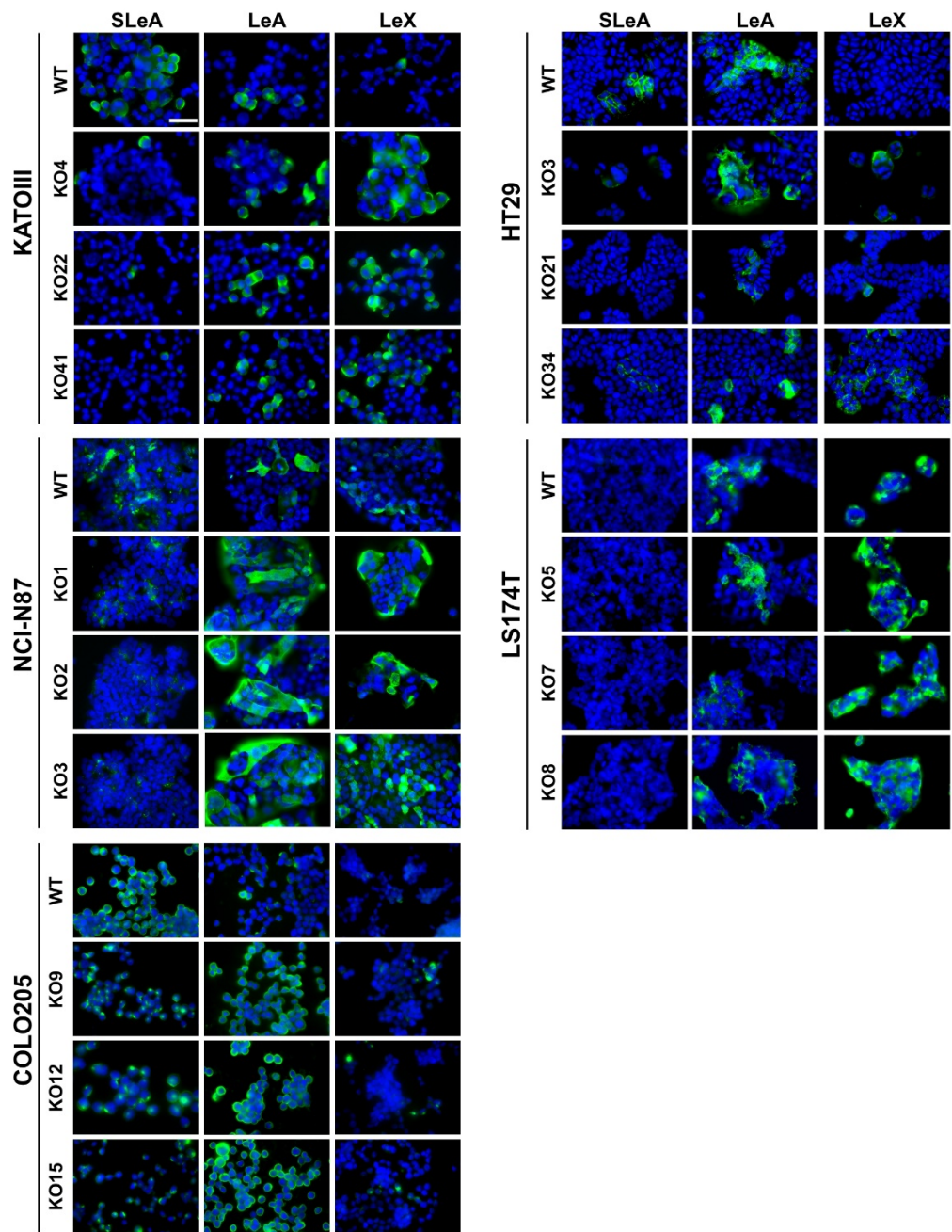


SFig. 2



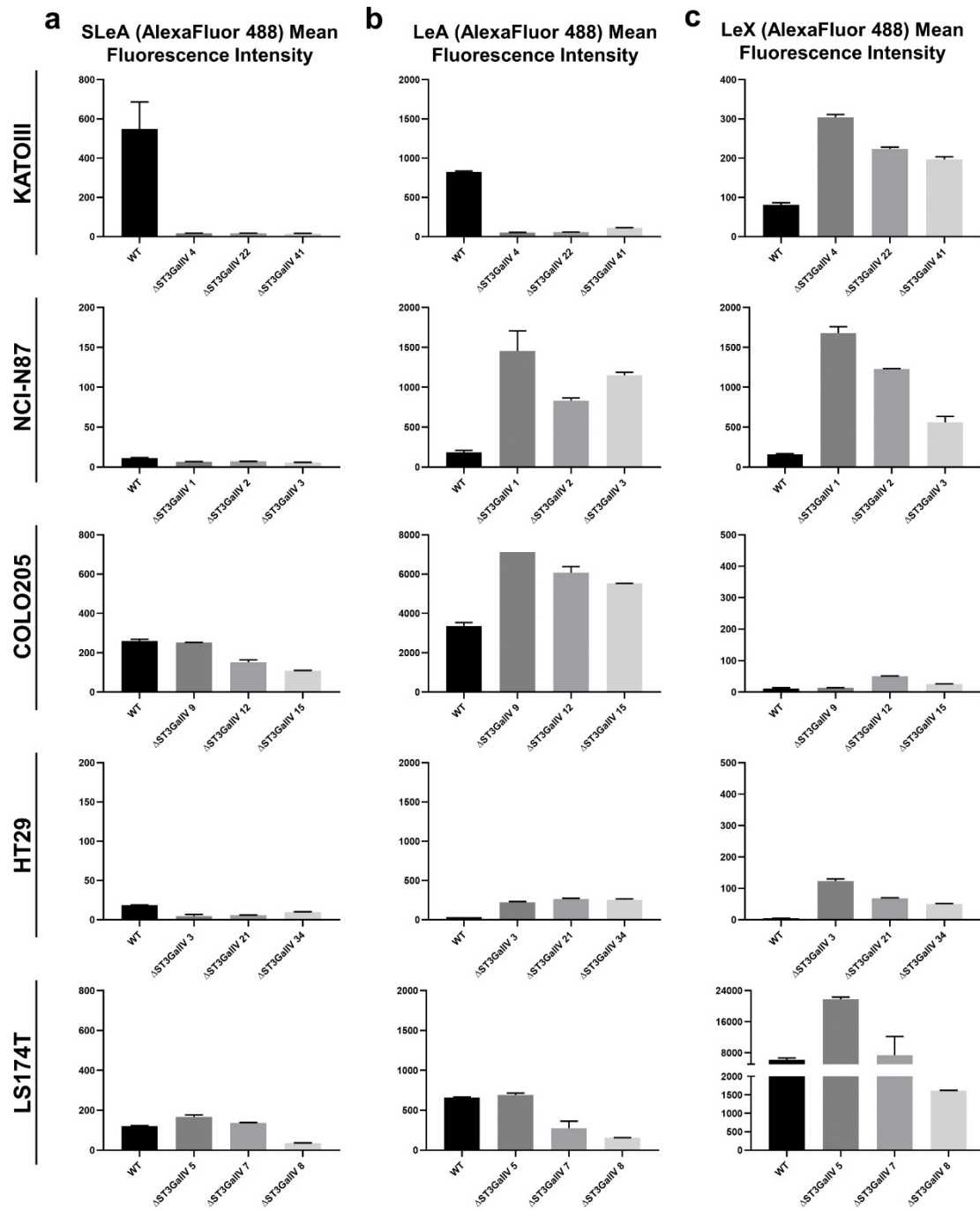
SFig.2: Mean fluorescence intensity levels of cell surface SLeX in WT and ST3GalIV KO clones of KATOIII, NCI-N87, COLO205, HT29 and LS174T cells. Intensity levels were quantified by flow cytometry analysis and shows the full abolishment of SLeX levels in the ST3GalIV KO cells. Bar graphs shows normalized mean intensity of fluorescence (MIF) + SEM, resulting from three independent biological experiments.

SFig. 3



SFig. 3: Immunofluorescence detection of SLeA, LeA and LeX in ST3GalIV KO GI cancer cells. Results showing a heterogenous expression of SLeA in the selected WT GI cancer cells with ST3GalIV KO leading to a reduced expression (left panel for each cell line). ST3GalIV KO also results in the increased expression of the Lewis precursors LeA and LeX (middle and right panel for each cell line). The scale bar corresponds to 50 μm.

SFig 4



SFig 4: Mean fluorescence intensity levels of cell surface SLeA (a), LeA (b) and LeX (c) in WT and ST3GalIV KO clones of KATOIII, NCI-N87, COLO205, HT29 and LS174T cells. Intensity levels were quantified by flow cytometry analysis. Bar graphs show normalized mean intensity of fluorescence (MIF) + SEM, resulting from three independent biological experiments.

Chapter 3

Monensin as a potential drug for treatment of SLeX-positive tumors

Ana F. Costa, Emanuel Senra, Diana Campos, Andreia Teixeira, Isabel Faria-Ramos, Liliana Santos-Ferreira, Sofia Lamas, Joana Gomes, Filipe Pinto, Rafaela Abrantes, Henrique O. Duarte, Mariana Pacheco, Marta T. Pinto, André F. Maia, António Pombinho, Rita Barros, Verónica Fernandes, Frederica Casanova-Gonçalves, Fabiana Sousa, José Barbosa, Luísa Pereira, Fátima Carneiro, Celso A. Reis and Catarina Gomes

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Monensin as a potential drug for treatment of SLeX-positive tumors

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Abstract

Colorectal (CRC) and gastric (GC) cancers remain the top lethal cancers and targeted therapies in this setting are still very limited. Sialyl Lewis X (SLeX), a cancer-associated glycan highly expressed in both CRC and GC, plays a crucial role in cancer cell dissemination and metastasis, thus presenting a promising but still underexplored therapeutic target. In this work, we performed a High-throughput screening (HTS) approach to identify potential inhibitors of SLeX expression in cancer cells. Two libraries including a total of 7836 compounds were screened and monensin emerged as a promising SLeX inhibitor. Monensin promoted structural alterations in the secretory pathway, particularly at the Golgi apparatus, impacting protein O-glycosylation and secretion. RNAseq transcriptomic analysis uncovered significant alterations in Gene Ontology (GO) terms associated with protein misfolding, target to the membrane, as well as epithelial cell-cell adhesion proteins. *In vitro* studies showed that, upon treatment with monensin, SLeX-positive cancer cells have reduced viability, concomitant with decreased motility and invasive capacities. *In vivo* cell line xenograft models of chick embryo chorioallantoic membrane (CAM) and nude mice revealed that monensin reduced tumor formation and invasion. Pre-clinical validation using GC patient-derived organoids (PDOs) and organoid xenotransplants in mice further underscored the clinical potential of monensin in suppressing the growth of SLeX-positive tumors. Overall, our findings set the ground for further evaluation of monensin as a novel therapeutic agent in GC and CRC in the clinical setting.

Keywords

SLeX, Glycosylation inhibitors, Gastrointestinal Cancers, High-Throughput Screening, Monensin

Introduction

The limitations of currently available cancer therapies frequently lead to tumor recurrence and metastasis, with the latter being, in fact, the major cause of mortality in cancer patients, accounting for approximately 90% of cancer-related deaths [1]. Gastrointestinal (GI) tract tumors, including gastric (GC) and colorectal (CRC) cancers, are major contributors to the current global landscape of cancer-associated mortality [2]. Therefore, it is of the utmost importance to identify novel anti-cancer agents with superior therapeutic efficacy.

A deeper understanding of the molecular changes that occur in neoplastic cells is key for drug discovery. Alterations in the glycosylation process are well-established features in cancer [3] and have been associated with tumor development, progression, therapy resistance, as well as cancer cell communication [4]. Particularly, increased expression of the Sialyl Lewis X (SLeX; Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc-R) antigen is commonly found in epithelial-derived tumors and has been associated with the malignant behavior of cancer cells and with more aggressive tumors [5-7]. Indeed, SLeX expression in numerous cancer types sustains tumor cell invasion and metastization, by facilitating tumor cell motility, adhesion to endothelial cells mediated by E-selectin binding, and interaction with platelets within blood vessels, promoting immune evasion and extravasation to distant sites [6,8,9]. Indeed, we have shown that the motility of glycoengineered GC and CRC cells depends on SLeX expression [10]. Additionally, the increased invasive capacity of GC cells, due to SLeX expression, was associated with the oncogenic activation of the c-Met signaling axis [7]. Therefore, SLeX holds promise as a therapeutic target, since its inhibition will lead to reduced tumor growth and metastatic dissemination.

The emergence of High-throughput screening (HTS) methodologies has provided a useful tool in the search for new therapeutic drugs. The application of such approaches to the discovery of glyco-specific drugs has been limited due to the complexity and spatiotemporal heterogeneity associated with the glycosylation process [11]. Nevertheless, recent advancements in methodologies have opened new possibilities [12,13]. For instance, an HTS assay, using reporters to specifically evaluate ppGalNAcT inhibitors, identified T3Inh-1 as a specific inhibitor of ppGalNAcT3. Moreover, a general inhibitor of glycosylation was identified by HTS using glycoengineered cells expressing tailored glycoproteins [14].

In this work, we aimed to develop an HTS approach specifically targeting the SLeX glycan antigen expressed in cancer cells. We identified a lead compound, monensin, a monovalent cation ionophore, as a potent inhibitor of SLeX expression and its associated malignant properties. In this study, the potential of monensin as an anti-cancer drug was further substantiated through *in vivo* xenografted models (CAM and mice) and the pre-

clinical model of GC patients-derived organoids (PODs). Overall, our findings set the ground for the use of monensin as a potential therapeutic strategy in the clinical setting.

Materials and Methods

Cell Culture and Cell lines

AGS, CACO-2, COLO205, KATOIII and RKO were obtained from ATCC (American Type Culture Collection) and MKN45 was obtained from the Japanese Cancer Research Resources Bank. Cells were grown in monolayer culture and maintained at 37 °C in an atmosphere of 5% CO₂, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX, HEPES medium, Dulbecco's Modified Eagle Medium (DMEM) or Iscove Modified Dulbecco Media (IMDM), all supplemented with 10% fetal bovine serum (FBS) (all from Biowest).

High-throughput screening of chemical compound libraries

COLO205 cells were seeded in 384-well plates (CellCarrier-384 Ultra, PerkinElmer) at a density of 1×10^5 cells/mL in RPMI medium supplemented with 10% FBS and allowed to adhere overnight. Two distinct libraries totalizing 7,836 chemical compounds (1,248 compounds from the Prestwick Chemical Library®, and 6,588 from DIVERSet® library) were then transferred, one compound per well, using a pintool (V&P Scientific) coupled to a MDT head of a JANUS Automated Workstation (PerkinElmer) for a final concentration of 4 µM in DMSO. Cells were incubated for 72 h, after which they were fixed with formaldehyde to a final concentration of 4% (Sigma Aldrich). SLeX was detected by immunofluorescence. Briefly, cells were incubated with CSLEX mouse antibody (BD Bioscience) overnight at 1:250 dilution, and fluorescent detection was performed using a sequential secondary amplification method of goat anti-mouse IgM and rabbit anti-goat Alexa 594 antibodies (Jackson ImmunoResearch), with 1 h incubation each. Nuclei were stained with DAPI (Sigma-Aldrich). The image acquisition was performed in an INCell Analyzer 2000 (GE Healthcare) with a Nikon 20x/0.45 NA Plan Fluor objective. Four fields of view (fov) were acquired per well. Image analysis was performed using the Ilastik and CellProfiler TM software [15,16]. The image analysis workflow consists in using the machine learning capabilities of Ilastik to accurately segment the nuclei. After, a custom-made pipeline in CellProfiler was devised to quantify the fluorescence intensity per cell obtained from the Texas Red (Alexa 594) channel. Briefly, upon correction of the uneven illumination, the probability maps obtained from the Ilastik software are used to identify the nuclei followed by a 3 pixels expansion of the edges of these objects to create a larger area, intending to reach the cell cytoplasm. Then, the mean pixel intensity of each fov in the Texas Red channel was calculated from the mean pixel intensity values of all identified cells on the image. The pipeline also generates outlines of the identified cells that were used to perform

quality control of the image analysis. To classify a compound as a hit we looked for a significative reduction or absence of Texas Red fluorescence, taking into consideration the number of cells per well to avoid false positives.

The Z'-factor of the assay was calculated according to the mean (μ) and standard deviations (σ) of samples (s) and controls (c), following the equations:

$$\text{Z-factor} = 1 - \frac{3(\sigma_s + \sigma_c)}{|\mu_s - \mu_c|}$$

Samples comprise COLO205 WT cells, whereas controls comprise COLO205 Δ ST3GalIV cells already described as SLeX negative [10].

Monensin dose response analysis

Hit confirmation was performed by testing the compounds in a dose-response assay (0nM – 500 nM) in triplicate. For monensin validation, cells were seeded in 96-well plates (CellCarrier-96 Ultra, PerkinElmer) and tested again in dose-response (repurchased compound, Sigma Aldrich) at a density of 3×10^4 cells/mL and allowed to grow for 24 h at 37°C and 5% CO₂ in a humidified incubator. Then, cells were incubated with monensin for 72 h and then fixed, stained, and analyzed, as previously described for the primary screen. DMSO was used as a negative control.

Flow Cytometry analysis

COLO205 and KATOIII cells were seeded in 6-well plates 24 h before treatment with increasing monensin concentrations for 72 h. On the day of analysis, cells were detached using trypsin (ThermoFisher), spin down at 300 g for 5 min at 4 °C. Afterwards, they were stained with CSLEX antibody (dilution 1:250, BDBioscience) for 30 min on ice. Cells were washed and incubated with secondary antibody Alexa 488 anti-mouse IgM (dilution 1:1000, Jackson ImmunoResearch) for 30 min on ice in the dark. Cells were stained with DAPI (10 μ g/ml, SigmaAldrich) for 10 min at room temperature in the dark. Samples were analyzed on a FACS Canto II Flow cytometer (BDBioscience) and *FlowJo* software of analysis.

MTT cell viability analysis

Cell proliferation/viability was analyzed *in vitro* by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using a commercially available kit (MTT Cell Proliferation Kit I, Roche). Cells were seeded into 96-well plates at the density of 5×10^3 cells/well, treated with monensin (or DMSO as control) and MTT Kit solutions added into each well according to cell proliferation kit instructions (Roche) for each time point 24, 48 and 72 h. The absorbance value was read at 600 nm using a microplate

reader. Three independent assays were performed in triplicates and results are presented as means $\pm$ SEM for each sample. The viability levels obtained were normalized and compared with the DMSO treatment control.

In addition, cell proliferation/viability was also assessed by seeding cells on a 24-well plate at the density of 2×10^4 in the late afternoon and were allowed to adhere overnight. In the next morning, cells were treated with monensin (or DMSO as control) and live and death cells were counted after staining with Trypan Blue (ThermoFisher) for three consecutive days (24, 48 and 72 h after treatment). The proliferation rate was calculated using a ratio between the cell number at the specific time after the treatment and the initial cell count.

Wound Healing Assay

Cell mobility was assessed by a wound healing assay *in vitro*. Approximately 5×10^5 cells were seeded into 24-well plates until reaching confluency. An incision was made in the central area with a 200- μ l pipette tip. After being washed twice with serum-free medium, the cells were then allowed to migrate into the cell-free area. The cells were imaged at 0, 24, 48 and 72 h at a magnification of 100x. Cell migration was calculated as the mean percentage of the cell migrated distance compared with the initial wound distance using ImageJ software (FIJI). The experiment was performed in triplicate in three biological replicates and motility capacity compared within the different conditions.

Invasion Assay

Invasion assays were performed in a BD Biocoat Matrigel invasion chamber (Corning) with an 8-mm diameter pore size membrane with a thin layer of Matrigel, in a 24-well plate. Inserts were rehydrated for at least 1 h with culture medium. After detachment of confluent cells with trypsin/EDTA, 9×10^4 cells were seeded in the upper surface of Transwell plates and cultured in: i) culture medium containing 10% FBS (control cells), ii) culture medium containing 10% FBS and DMSO (control treatment cells, final concentration of 0,002%), iii) culture medium containing 10% FBS and 100 nM and 200 nM of monensin for COLO205 cells and 50nM for KATOIII cells (treatment cells), all during 72h in a humidified incubator (37°C, 5% CO₂). Complete culture medium (media with 10% FBS) was added in the lower part of the insert. After incubation, non-invading cells in the upper part of the insert were carefully removed with a cotton swab, invasion chambers washed with PBS and lower membrane penetrated cells fixed with methanol. Membranes were removed from the inserts and mounted in a slide using Vectashield with DAPI (Vector labs). Two independent assays were performed, and cells were seeded in duplicate for each condition. Invading cells were counted under a fluorescence microscope (Carl Zeiss), and measurement was done by

counting cells, using ImageJ software, from six random fields using a microscope at a magnification of 200x. Results are presented as means $\pm$ SEM for each sample, and invasion levels obtained were compared within the different conditions.

Transmission electron microscopy analysis

For the ultrastructure analysis, cells were fixed in a solution of 2.5% glutaraldehyde (Electron Microscopy sciences) with 2% formaldehyde (Electron Microscopy sciences) in 0.1 M sodium cacodylate buffer (pH 7.4) for 2hrs, at RT, and post fixed during 1hr in 1% osmium tetroxide (Electron Microscopy Sciences) diluted in 0.1 M sodium cacodylate buffer. After centrifugation, the pellet was resuspended in HistogelTM (ThermoFisher Scientific) and then stained with aqueous 1% uranyl acetate solution overnight, dehydrated and embedded in Embed-812 resin (Electron Microscopy sciences). Ultra-thin sections (50 nm thickness) were cut on an RMC Ultramicrotome (PowerTome) using Diatome diamond knives, mounted on mesh copper grids (Electron Microscopy Sciences), and stained with uranyl acetate substitute (Electron Microscopy Sciences) and lead citrate (Electron Microscopy Sciences) for 5 min each. Samples were viewed on a JEOL JEM 1400 transmission electron microscope (JEOL), and images were digitally recorded using a CCD digital camera Orius 1100W. The transmission electronic microscopy was performed at the HEMS core facility at i3S, University of Porto, Portugal with the assistance of Ana Rita Malheiro, Sofia Pacheco and Rui Fernandes.

RNASeq analysis

Cells were seeded in 6-well plates and allowed to seed for 24 h. Then, cells were treated with Monensin or DMSO (vehicle) for 72 h. Cells were harvested and RNA extracted using Pure LinkTM RNA Mini Kit (Invitrogen) according to manufacturer protocol. The quality of the RNA was first evaluated by NanoDropTM One Spectrophotometer (ThermoFisher Scientific) and the samples were stored at -20°C until use. Three biological replicates from each condition were guaranteed. The RNA was sent to Novogene Co., Ltd. for cDNA library construction, sequencing and bioinformatic analysis. The quality of the paired-end sequencing reads was checked using FastQC and the low-quality reads and adapters were removed using Trimmomatic. The pre-processed reads were mapped to the reference using HISAT2. The resulting read alignments were then used to generate the gene counts using the feature Counts software and normalised to FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced). Differential gene expression (DEG) analysis of the gene counts data was performed using DESeq2 R employing a differential gene screening threshold of $|\log_2(\text{FoldChange})| \geq 1$ & $\text{padj} \leq 0.05$.

Through the enrichment analysis of the differential expressed genes, the enrichment analysis on gene sets (GO) was obtained using the clusterProfiler software.

Immunofluorescence analysis

Cells were seeded in 13 mm coverslips (Marienfeld) and let adhere overnight. Then, the cells were treated with Monensin or DMSO for 72 h. The cells were washed with PBS and fixed with 4% Paraformaldehyde fixative solution (Alfa Aesar) for 10 minutes at room temperature. Blocking of immunoglobulins cross-reaction was performed using goat serum in 10% BSA in PBS at room temperature for 30 minutes, followed by the primary antibody incubation at 4°C overnight. The primary antibodies used were: SLeX (CSLEX, 1:100, BD Bioscience,), Tn (1E3, 1:2, Hybridoma), E-Cadherin (4A2C7, 1:200, Invitrogen), Calnexin (C5C9, 1:100, Cell Signaling), ERGIC (LMAN1, 1:100, Cusabio), Giantin (ab24586, 1:1000, Abcam) and TGN46 (ab50595, 1:300, Abcam). Afterwards, incubation with fluorescently labelled secondary antibodies anti-mouse IgM Alexa Fluor® 594 (1:500) or IgG Alexa Fluor® 488 (1:500), or anti-rabbit IgG Alexa Fluor® 488 (1:500, ThermoFisher Scientific) were performed during 1 h at room temperature and nuclear counterstaining performed using 4',6'-diamino-2-phenyl-indol (DAPI) for 10 minutes. Washes were performed with PBS. The fluorescent signal was examined using a fluorescence microscope and images were acquired using a Zeiss Axio Carl cam MRm and the AxioVisionRel (version 4.8) analysis software.

Protein extraction of cellular fractions for downstream analysis

For extraction of total, cytosol, and membrane proteins, COLO205 and KATOIII were cultivated in a 15-cm dish and let adhere overnight, and treated with Monensin or DMSO for 72 h. The commercially available kit Pierce™ Cell Surface Biotinylation and Isolation Kit (ThermoFisher) that allow for selective biotinylation, solubilization, and enrichment of plasma membrane proteins was used. For the total protein, after the cell's lysis step, a small amount of protein was collected. For the cytosol protein, during the isolation labeled proteins step, the flowthrough was collected as it corresponds to non-surface and non-biotinylated proteins. For the enriched membrane protein lysates, in the elution step, a 0,1% Rapigest and 10mM DTT in 50 mM ammonium bicarbonate buffer was used.

For secreted protein enrichment, COLO205 and KATOIII were cultivated in T75 and let adhere overnight. The cells were washed five times in PBS 1X and treated with Monensin or DMSO, diluted in serum-free culture medium for 72 h. The conditioned media (secretome) was collected and centrifuged for 5 min at 1200 rpm to remove cell debris and concentrated using 10 kDa Amicon centrifugal filter units (Merck Millipore, Burlington, MA,

USA). 4 μ L of complete TM protease inhibitor cocktail (Roche) was added per 100 μ L of concentrated samples and then stored at -80°C .

SDS-PAGE and Western Blot Analysis

After protein concentration quantification through bicinchoninic acid protein assay (BCA) (Pierce), 5 μ g (COLO205) and 15 μ g (KATOIII) of protein extracts were loaded onto 4-15% Mini-PROTEAN®TGXTM precast polyacrylamide gels (Bio-Rad) for electrophoretic separation (SDS-PAGE) (Bio-Rad). Gels were then transferred onto nitrocellulose membranes (Amersham) and blocked for 1 h with 5% bovine serum albumin (BSA) (Sigma-Aldrich) diluted in PBS containing 0.05% Tween 20 (Sigma-Aldrich) (PBS-T). For Western blotting, the membranes were incubated overnight at 4°C with SLeX (CSLEX, 1:500, BD Bioscience) primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgM (1:15000, Jackson ImmunoResearch). Protein bands were visualized using the ECL WB detection reagent and ChemiDoc Imaging System (Bio-Rad).

Chicken embryo in vivo tumorigenesis assay

The chicken embryo chorioallantoic membrane (CAM) model [17]. was used to evaluate the angiogenic response and growth capability of COLO205 cells treated with different concentrations of monensin compared to DMSO control ($n = 13$ for each group). Briefly, fertilized chick (*Gallus gallus*) eggs obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5–2 mL of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. The window in the eggshell does not interfere in any way with the normal development of the chick embryo. COLO205 cells were resuspended in a solution containing monensin 200 nM or DMSO 0.01% (control) and vitrogel (TheWellBioscience). The cells' containing solution (2×10^6 cells per embryo) were placed on top of E10 growing CAM into a 3 mm silicon ring under sterile conditions. The eggs were re-sealed and returned to the incubator for an additional 6 days. The embryos were euthanized by adding 2 mL of fixative to the top of the CAM which is a very efficient and fast method. After removing the ring, the CAM was excised from the embryos, photographed *ex ovo* under a stereoscope, at 20x magnification (Olympus, SZX16 coupled with a DP71 camera). The area of CAM tumors was determined using the Cell A (Olympus) software. Evaluation of tumor invasion was performed in a blind fashion by two independent observers. The semi-quantitative evaluation took into consideration the quantity of human cytokeratin AE1/AE3 labeled cells present in the CAM mesenchyme.

According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs.

Establishment of COLO205-LUC cell line and xenograft tumors of human cancer cells

COLO205-LUC were obtained by lentivirus transfection of the COLO205-LUC with the CMV-Luciferase (firefly)-2A-GFP (Puro) viral particles (LVP020, Gentarget) containing the bioluminescent reporter protein luciferase. Cells were maintained in RPMI supplemented with puromycin (1 µg/ml) (InvivoGen). Approximately 1×10^6 or 5×10^6 of COLO205-LUC cells were injected subcutaneously into the dorsum of 8 to 10-week-old female N:NIH(s)II:nu/nu nude mice, aged 8–10 weeks and weighing approximately 20 g (i3S animal facility, Portugal). At day 7, when the tumor volume reached between 50-100 mm³ (in 1×10^6 cells inoculum) or 200-250 mm³ (in 5×10^6 cells inoculum), the COLO-205 LUC xenograft mice were randomized into 2 groups (n=5 to 6 mice per group) and treated intraperitoneally (IP) with 100 µL of saline solution/PBS (control group) or Monensin (10 mg/kg body weight) every two days. Monensin was dissolved in 100% ethanol to prepare a 40 mg/ml drug storage solution. *In vivo* luminescence imaging using the Ivis Lumina series III (PerkinElmer) at 4, 7, 14 and 21 days after cell injection. Animals were anesthetized with 5% (V/V) isoflurane for induction and 1–2% (V/V) for IVIS acquisition. During the treatment period, the tumor volume was measured every other day with a caliper and calculated as $TV = (a \times b \times c) \text{ mm}^3$ (Longest axis - a, shortest axis - b, and thickness - c). Animals were sacrificed, and the tumor and organs were collected after 21 days of pos-cell injection. All animal assays were conducted in strict conformity with the guidance of the European Union Directive 2010/63/EU, following a protocol previously authorized by the i3S Animal Ethics Committee and the Competent Authority in Portugal (Direção-Geral de Alimentação e Veterinária (DGAV)) (reference 2021_05) regarding the humane endpoints, appropriate husbandry and protection of experimental animals. Mice were bred, cared and fed *ad libidum* at the i3S Animal Facility (Porto, Portugal).

Patient-Derived Tumor Organoid viability assay

Gastric tumor patient-derived organoids (PDOs) were previously established by the group. For this study, four tumoral PDOs were used to assess the effect of monensin on tumor growth. Monensin dose-response in patient-derived tumor organoids was assessed using the CellTiter-Glo® 3D Reagent (Promega Corporation). For that, 400 organoid fragments/well were resuspended in 20 µL of matrigel and seeded in a 96-well plate. After one day post seeding, organoids were treated with 0-400 nM of monensin for 6 days and the compound was renewed every 3 days. Upon treatment, the medium of each well was

removed and 80 μ L of fresh simple medium was added followed by 100 μ L of CellTiter-Glo® 3D Reagent. Tumor organoids were mechanically disrupted by up-and-down using a pipette and incubated for 25 min at RT. Luminescence signal was quantified through a SynergyMx™ MultiMode Microplate Reader (BioTek™). Viability values were normalized to the medium only-control (0 nM) as 100 % activity.

Patient-Derived Tumor Organoids immunofluorescence analysis

Free floating fixed organoids were permeabilized with 50 mM of NH₄Cl for 10 min and with PBS 0.3% Triton X-100 (Sigma Aldrich) for 15 min in rotation followed by washing steps using PBS 0.3% Triton X-100. Next, samples were blocked with the UltraVision Protein Block for 15 min followed by the primary antibody incubation with SLeX (CSLEX, 1:250, BD Bioscience) at 4°C overnight in rotation. After that, organoids were incubated for 45 min in rotation at room temperature with fluorescently-labeled secondary antibodies anti-mouse IgM Alexa Fluor® 594 (1:500, ThermoFisher Scientific). Nuclei were counter stained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich). Finally, organoids suspension was visualized using the inverted microscope Leica DMI6000-CS (Leica Microsystems, Germany). Images were acquired using the software LAS AF (Leica Microsystems, Germany).

Subcutaneous implantation of Patient-Derived Tumor Organoids Xenograft (PDOX)

To develop gastric Patient-Derived Tumor Organoid Xenograft, female N:NIH(s)II:nu/nu nude mice, aged 8–10 weeks went through complete anesthesia by isoflurane 5% (V/V) exposure followed by a continuous 1.5-2% (V/V) isoflurane flow. Previously, before anesthesia, buprenorphine (0.08 mg/Kg) was subcutaneously injected for pain management. Then, a small incision was made in the upper middle part of the dorsal area and a subcutaneous “pocket” was created by blunt dissection. A 50 μ L suspension of a determined/ chosen organoid dissolved in a matrigel matrix/PBS mixture with approximately 8000 to 12000 organoids was injected (resorting to a 200ul slashed pipette tip), into the “pocket”. Using a PGA nylon silk (Silkam), two or three stitches were done to close the surgical incision. Mice were daily monitored, and Paracetamol was added to the drinking water for 72 h after surgery. Mice were weighed and monitored for tumor development every other day. The longest axis (a), shortest axis (b), and thickness (c) of each tumor were measured using a digital caliper and the total volume was calculated. Tumor growth curves were constructed using the average tumor areas. When tumors reached 100-150mm³, the mice were divided into two groups and IP treated with PBS (Control group) and monensin (10 mg/kg body weight) every two days, or four groups where PBS, monensin (10 mg/kg body weight), 5FU (10 mg/kg body weight) and monensin + 5FU

administered three days a week. Once tumors achieve a max 2000 mm³ or when they reach 100 days after surgery, the tumors were collected and fixed in 10% buffered formalin and embedded in paraffin. A histological analysis was performed using hematoxylin and eosin (HE) staining.

Immunohistochemistry analysis

Excised CAMs and xenograft mice tumors and organs were fixed in 10% neutral-buffered formalin, paraffin-embedded for slide sections and stained with Hematoxylin and Eosin (H&E for histological examination) and immunostained for cytokeratin and SLeX detection to characterize the phenotype of CAM and xenograft mice tumors. Briefly, sections were dewaxed, rehydrate and the endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 30 min. Then, antigen retrieval was achieved with citrate buffer pH 6, and sections were incubated with animal-free blocker (Vector Laboratories) for 1h. For CAM tissues, a goat anti-chicken IgY antibody (1:100, Abcam) was added to the blocking solution to reduce unspecific staining. Then, incubation with the monoclonal antibodies SLeX (CSLeX, 1:350, BD Biosciences), cytokeratins (AE1/AE3 1:3000 (CAM tissue) and 1:500 (mouse tissue), CellMarque) and Ki67 (D3B5, 1:500, CellSignaling) was performed overnight at 4°C, followed by biotinylated polyclonal rabbit anti-mouse immunoglobulins (DAKO) 30min incubation at room temperature and avidin/biotin complex detection (Vectastain). Finally, all samples were stained with 3,3-diaminobenzidine tetrahydrochloride (SigmaAldrich) containing 0.02% H₂O₂ and counterstaining of the nucleus was done with Mayer's hematoxylin (Empredia).

Statistical Analysis

Data was analyzed with GraphPad Prism 8.0 software (CA, USA). Quantitative data were expressed as the mean ± SEM of three biological replicates. The level of significance was assessed using Student's t-test and one or two-way ANOVA. p - values of <0.01 were considered significant.

Results

Fluorescent High-throughput screening for SLeX inhibitors identifies monensin as the lead compound

The SLeX-positive CRC cell line COLO205, known for its 100% positivity for SLeX expression and monolayer growth pattern [10], was used for the HTS, facilitating fluorescent image acquisition and accurate data analysis. We screened 7836 compounds comprising two distinct libraries, the Prestwick Chemical library® and the DIVERSet® library (Fig 1A and Fig S1). As our inhibitory readout relies on glycosylation turnover at the cell membrane, an

optimized 72 h incubation period was applied. We developed a custom pipeline in CellProfiler™ to quantify the mean fluorescence intensity per cell obtained from the Texas Red (Alexa 594) channel. Given the heterogeneity in SLeX expression within the selected cell line, mean intensities within the four fields of view (fov) exhibited considerable standard deviations, resulting in a Z'-factor of 0.539 (Fig 1B). Compounds that induced a significant decrease in fluorescent signal without causing a substantial loss of cell number (indicative of toxicity) were considered HIT compounds.

Most of the tested compounds from our screening assay showed no significant impact on SLeX expression (Fig 1C). Interestingly, while some compounds resulted in increased SLeX levels, a few led to decreased SLeX expression. Manual image analysis revealed that, although some compounds seem to induce a higher SLeX expression level, the observed intensity signal was due to cell debris and, therefore, considered a false result. The Prestwick Chemical library® yielded better results, rendering 4 HIT candidates: glimepiride, auranofin, lanatoside C, and monensin. According to our HIT selection criteria, which considered HITs with less than 200 cells per well as false positives, monensin fulfilled all the defined criteria (Fig 1C and Fig S1) and therefore, was selected for the subsequent validation assays.

Monensin decreases SLeX expression on the surface of cancer cells

The inhibitory capacity of monensin towards SLeX expression was validated in COLO205 through a dose-response manner, using concentrations ranging from 0 to 500 nM and the same fluorescent detection assay employed in the HTS. The results demonstrated that monensin effectively inhibits SLeX expression at the cell surface, with concentrations of 100 and 200 nM significantly reducing SLeX expression without compromising cell viability (Fig 1D and E). To further validate monensin's inhibitory capacity, we assessed SLeX expression on COLO205 by flow cytometry in a dose-dependent manner. COLO205 cells exhibited decreased SLeX expression at the cell surface when treated with monensin concentrations above 100 nM, highlighting its promising inhibitory effect (Fig 1F). This inhibitory effect was also validated in the KATOIII GC cell line, with flow cytometry results revealing a dose-dependent inhibition of SLeX expression (Fig 1G). Overall, these findings underscore monensin's potential as a promising drug to inhibit SLeX-associated malignant features of CRC and GC cells.

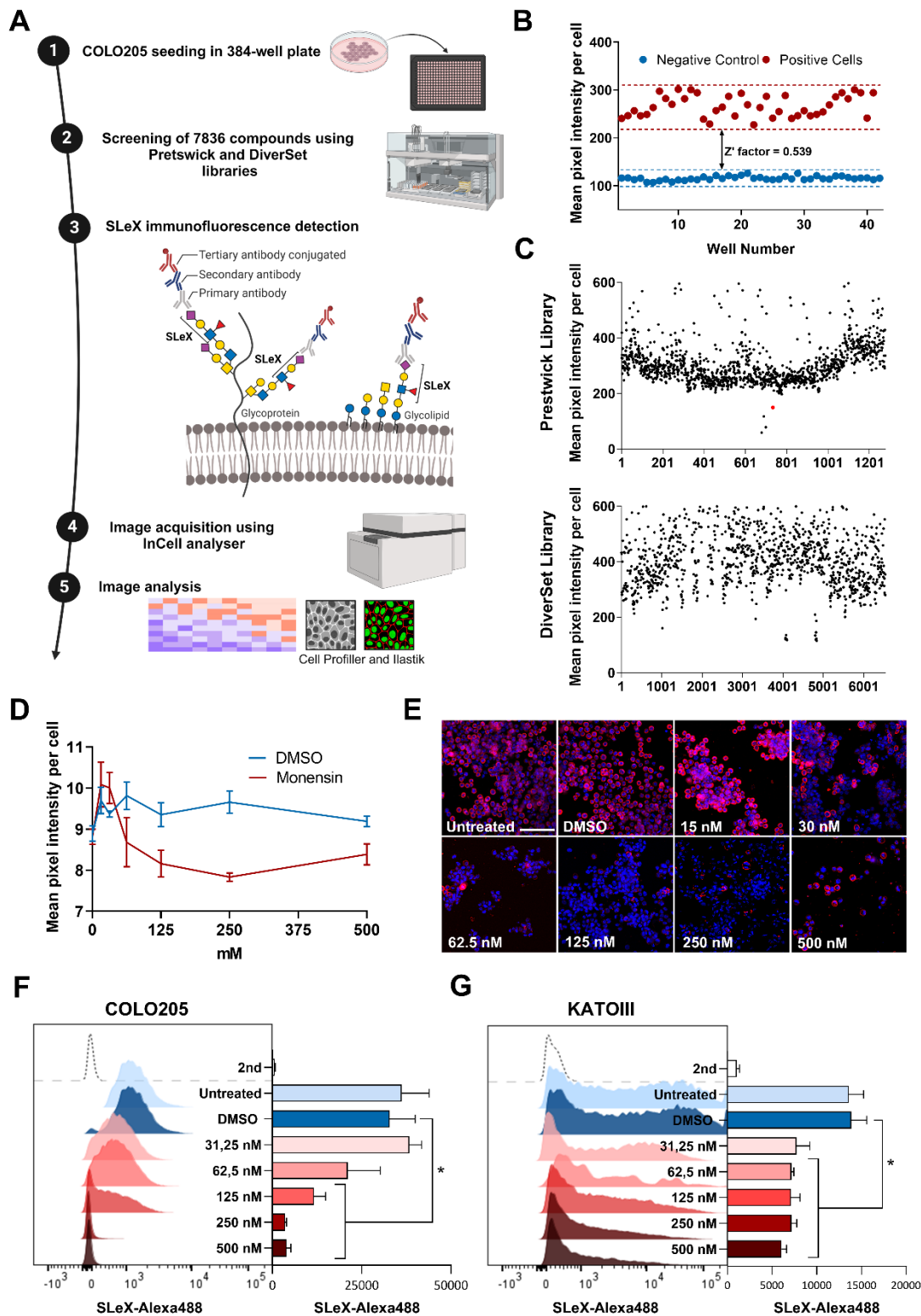


Figure 1: HTS approach identifies monensin as a promising SLeX inhibitor. A) Schematic representation of the adopted methodology involved in the HTS setup. **B)** Z'-factor determination of HTS assay in 384-well plates considering the detection of SLeX in COLO205 WT cells as the positive control and COLO205 Δ ST3GalIV as the negative control. Values of Z' higher than 0,5 allow us to proceed with confidence with the HTS assay. **C)** Graphic representation of SLeX detection in each well containing the different screened compounds from two distinct libraries (Prestwick® and DIVERSet®). Monensin (red dot) was one of the identified hits, according to

the selection criteria. **D)** Graphic representation of SLeX immunodetection at the cell surface of COLO205 cells treated with DMSO (blue) or with different monensin concentrations ranging from 0 to 500 nM (red), and **E)** representative images of immunofluorescence staining showing the reduction in SLeX immunodetection in the different tested monensin concentrations in a dose-dependent manner. The scale bar corresponds to 100 μ m. **F) and G)** SLeX immunodetection by flow cytometry in COLO205 cells (**F)** and KATOIII cells (**G)** upon treatment with DMSO (blue) or monensin (red), validating the inhibitory capacity of monensin in a dose-response manner. * $p < 0.01$ vs. the DMSO-treated group.

Monensin impacts Golgi morphology and function, affecting protein glycosylation and processing

Treatment of CRC and GC cells with monensin led to marked alterations in the cellular morphology (Fig S2A). Monensin-treated cells exhibited larger vacuoles and increased cellular aggregation, indicative of a phenotype associated with cytosolic stress. Transmission electron microscopy (TEM) analysis was employed to investigate potential alterations induced by monensin treatment regarding cytoarchitecture and organelle morphology. TEM analysis of monensin-treated cells revealed the increased presence of autophagic and Golgi-associated vacuoles indicative of Golgi stress in both COLO205 and KATOIII cells (Fig 2A). Additionally, we identified altered mitochondrial morphology in monensin-treated GC cells (Fig 2A).

We further explored the molecular mechanisms associated with monensin treatment in COLO205 and KATOIII cancer cells using RNA sequencing transcriptomics. Gene ontology (GO) enrichment analysis of monensin-treated cells compared to DMSO-treated uncovered significant alterations in pathways primarily associated with i) endoplasmic reticulum (ER) unfolded protein response (UPR), ii) response to misfolded proteins, iii) protein targeting to the membrane, and iv) cell adhesion molecule binding (Fig 2B). Indeed, the epithelial cell-cell adhesion protein E-cadherin was shown to have altered levels of expression (Fig S2B) and mislocalization in the cytosol (Fig 2C), particularly in COLO205 cancer cells, validating monensin's impact on the expression of adhesion molecules. Moreover, although some SLeX immunodetection persisted in monensin-treated cancer cells, it was mainly confined to a Golgi-like pattern of expression (Fig 2C), indicative of protein retention within cells. Considering that protein glycosylation, mainly O-glycosylation, occurs at the Golgi apparatus and is essential for proper protein folding, secretion and function, we explored the impact of monensin on the secretory pathway and SLeX biosynthesis. Our findings revealed differences in the pattern and levels of expression of ER, ER-Golgi intermediate compartment (ERGIC) and Golgi markers, highlighting significant reduction and diffused expression in ERGIC and trans-Golgi (Fig 2D). Additionally, treatment with monensin leads to the increased expression of the clathrin endocytosis marker. Importantly, we found that monensin disrupted the proper O-glycan

biosynthesis, leading to the expression of Tn glycan, the first glycan structure on the O-glycan synthesis (Fig 2D). This shows that the O-glycan biosynthesis is disrupted in the early elongation steps, hampering the synthesis of more complex structures such as SLeX. As an outcome, we observed a reduction in cell membrane and secretion of SLeX-positive proteins (Fig 2E).

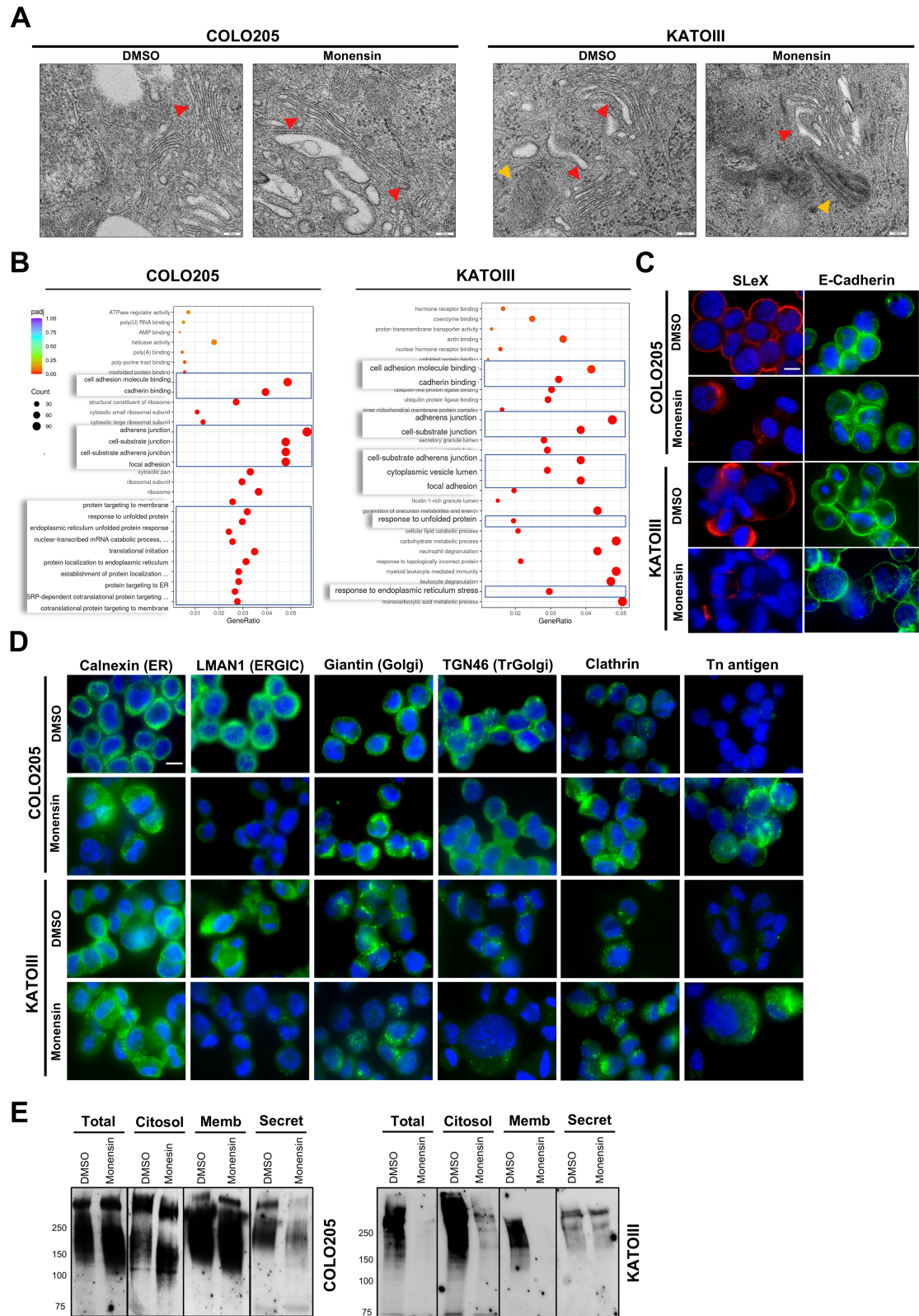


Figure 2: Molecular impact of monensin in COLO205 and KATOIII cell lines. **A)** Transmission electron microscopy (TEM) analysis, showing morphological alterations mainly at the Golgi apparatus (red arrow) and mitochondria (yellow arrow) upon monensin treatment. Images were acquired at a 12000-20000X magnification. **B)** Gene ontology enrichment analysis from RNA sequencing (RNA-seq) revealing that monensin treatment led to transcriptomic alterations associated with endoplasmatic reticulum (ER) stress, protein folding and targeting to the cell membrane and adhesion. **C)** Immunodetection of SLeX (left panel), and E-cadherin (right panel) in COLO205 and KATOIII cell lines upon treatment with DMSO or monensin displaying a reduction and mislocalization in SLeX and E-cadherin expression. **D)** Immunofluorescence analysis of molecular markers of protein processing cellular compartments showing alterations in the expression and localization of calnexin (ER), LMAN1 (ERGIC), Giantin (Golgi), TGN46 (TransGolgi), Tn antigen and Clathrin (endocytosis). Images were acquired at 630X magnification, and the scale bar corresponds to 10 μ m. **E)** Western blot analysis of SLeX expression in different cellular compartments (total, cytosolic, membrane, and secreted) in COLO205 (right) and KATOIII (left) cells treated with DMSO or monensin, demonstrating a reduction in cell membrane and secretion of SLeX-positive proteins.

Monensin undermines SLeX-induced malignant features in CRC and GC cells

Given monensin's ability to reduce SLeX expression on the cancer cell surface, we comprehensively assessed its biological impact and therapeutic potential. We observed that monensin treatment affected the metabolism of SLeX-positive cancer cells COLO205 and KATOIII (Fig 3A). Notably, COLO205 cells showed a statistically significant increase in cellular metabolism at 24 h and 48 h when subjected to both 100 and 200 nM monensin concentrations. However, this effect was not evident at 72 h although higher concentration (500 nM) of monensin negatively impacted cellular metabolism. Conversely, in KATOIII cells, concentrations superior to 100 nM negatively affected cellular metabolism after 48h of treatment. Remarkably, no significant effects were observed in the panel of SLeX-negative CRC and GC cells (Fig 3A).

Subsequently, we investigated whether the observed monensin-induced metabolic alterations resulted in variations in cell proliferation and cell death. We observed that in COLO205 cells, concentrations ranging from 25 to 200 nM did not have an impact on cell proliferation and cell death (Fig 3B and C). An exception was noted at 500 nM concentration, which induced a substantial reduction in proliferation and an increase in cell death, indicative of cellular toxicity. Regarding KATOIII cells, concentrations higher than 50 nM resulted in a significant reduction in proliferation rates at 72 h (Fig 3B). Similarly, concentrations above 50 nM led to a significant increase in cell death after 24h or 48h, in particular with the 500 nM concentration (Fig 3C), suggesting its potential as a killing agent.

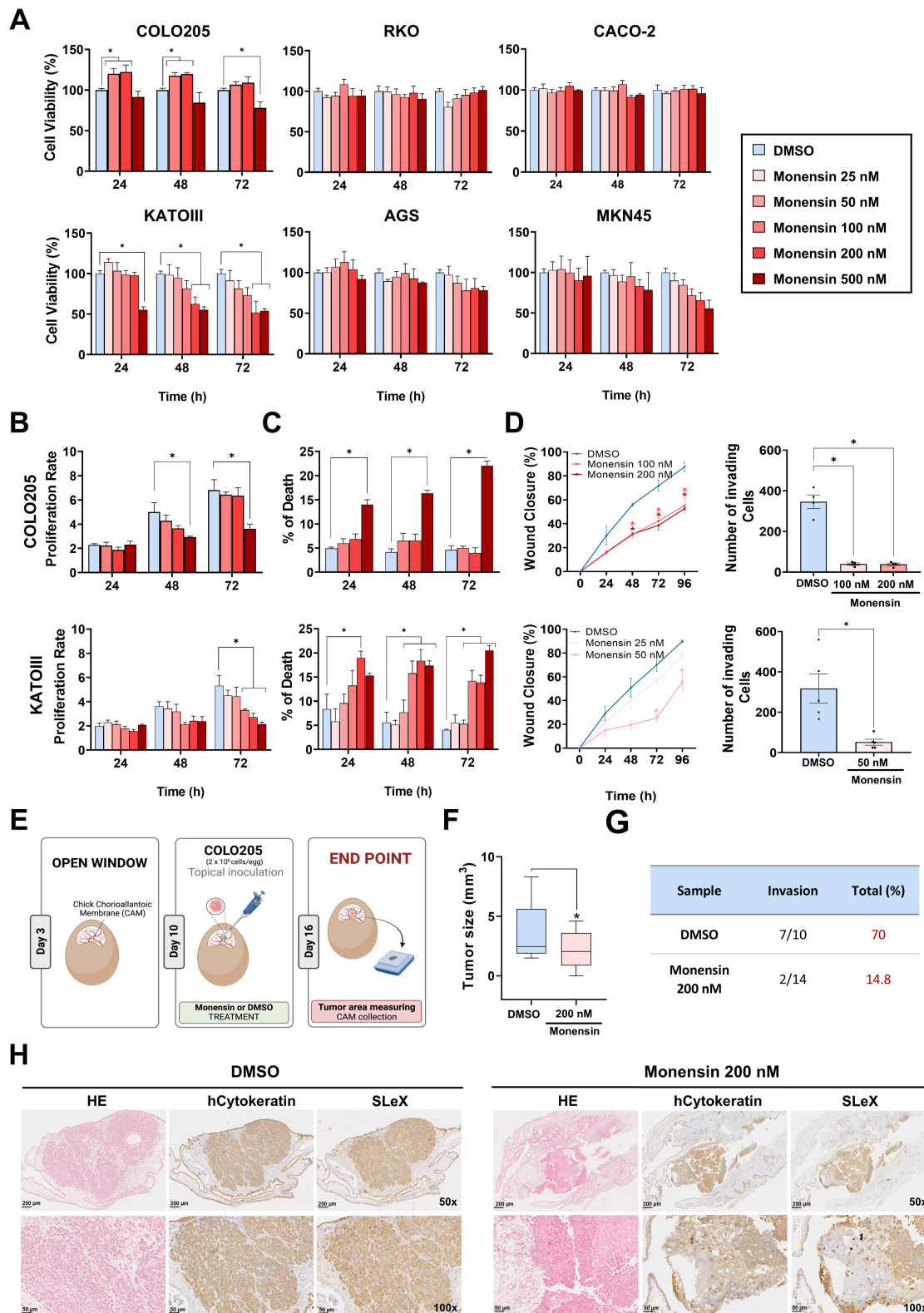


Figure 3: Effect of monensin on cancer cell metabolism, death, proliferation and invasion. A) Evaluation of cell metabolism through the MTT assay in SLeX-positive (COLO205 and KATOIII) and SLeX-negative cancer cell lines (RKO, CACO-2, AGS and MKN45), showing that monensin can significantly impact cell viability only in SLeX-positive cells **B)** Proliferation rate of COLO205 and KATOIII cancer cells, measured along 72h of treatment, demonstrating monensin's inhibitory activity at higher concentrations **C)** Percentage of cell death in COLO205 and KATOIII cancer cells treated with monensin for 72 h, showing increased cell death mostly in

KATOIII cells. **D)** Wound healing (Left) and invasion (Right) assays, showing monensin's ability to reduce COLO205 and KATOIII migratory and invasive properties. **E)** Schematic representation of the workflow adopted for the *in vivo* study of the effect of monensin on tumor formation and invasion using the chick chorioallantoic membrane (CAM) model. **F)** COLO205 derived xenografted tumor area, showing the development of smaller tumors in monensin-treated CAM (Right) when compared to DMSO treated CAM (Left). All results are presented as mean $\pm$ SEM of at least three independent experiments. * $p < 0.01$ vs. the DMSO-treated group. **G)** Number of invading xenografted COLO205 derived tumors, showing monensin's ability to efficiently reduce tumor cell invasion into the CAM when compared to DMSO-treated cells. **H)** Representative images of immunohistochemical analysis of CAMs, including Hematoxylin & Eosin (H&E) staining, human cytokeratins and SLeX immunodetection. Cytokeratins immunostaining depicting the reduced invasive capacity of monensin-treated cells in the CAM, concomitant with reduced SLeX expression.

Given the known association of SLeX expression with cancer cell invasion and the presence of metastasis in CRC and GC [6,9], we assessed monensin's ability to impair cell motility and invasion. To this end, COLO205 and KATOIII cancer cells were treated with monensin for 72 h and a wound healing and Matrigel-chamber invasion assay were performed. Monensin concentrations that showed significant differences in cell proliferation and cell death were not considered in these assays. Our results revealed that both COLO205 and KATOIII cells treated with monensin displayed a decreased capacity to migrate and invade when compared to DMSO-treated cells (Fig 3D). These results underscore the potential of monensin to impair SLeX-associated motility and invasive capacities *in vitro*.

To further elucidate the *in vivo* effect of monensin, we employed the chick chorioallantoic membrane (CAM) model to evaluate the growth and metastatic potential of both KATOIII and COLO205 cells. KATOIII cells presented a diffuse growth pattern, thus an accurate measure of the CAM xenografted size is not possible (Fig S3A). Given that only COLO205 cells formed measurable tumors, this was the cell model analyzed. Tumor formation was measured 6 days post-cell inoculation on the top of the CAMs (Fig 3E). Results showed that monensin-treated cells formed significantly smaller tumors (Fig 3F). In addition, DMSO-treated cells exhibited significantly higher invasive capacity (70%) than monensin-treated cells (14,8%), as evidenced also by H&E staining. Additionally, IHCs staining showed reduced SLeX expression in monensin-treated cells. (Fig 3G and H and Fig S3B). Collectively, these results underscore the ability of monensin to reduce SLeX expression in cancer cells and mitigate their associated malignant properties *in vivo*.

Monensin impacts tumor growth in mice

To further substantiate the *in vivo* potential of monensin in inhibiting SLeX-driven malignancy, we assessed monensin's therapeutic efficacy in a COLO205 xenograft mouse model. Thus, 1 and 5×10^6 COLO205 cells were subcutaneously inoculated into nude mice, and mice were IP treated after day 7 with monensin (10 mg/kg) or PBS. Tumor burden and SLeX expression were then analyzed (Fig 4A). The results showed that monensin significantly inhibited tumor growth, irrespective of cellular inoculum (Fig 4B and C, Fig S4A). In addition, SLeX immunodetection in collected monensin-treated tumors was significantly decreased (Fig 4D and Fig S4B and C). Notably, COLO205 xenografts exhibited rapid tumor growth, leading to ulceration within a few days (around day 10 after starting treatment). Quantification of tumor ulceration revealed positive outcomes in monensin-treated mice compared to untreated tumors, that exhibited a higher percentage of ulceration (Fig 4E). Tissue analysis of collected organs did not show any metastatic lesion in any group of mice (Fig S4D), which can be attributed to the relatively short duration of this experiment. Taken altogether, monensin is shown to effectively control tumor growth *in vivo*.

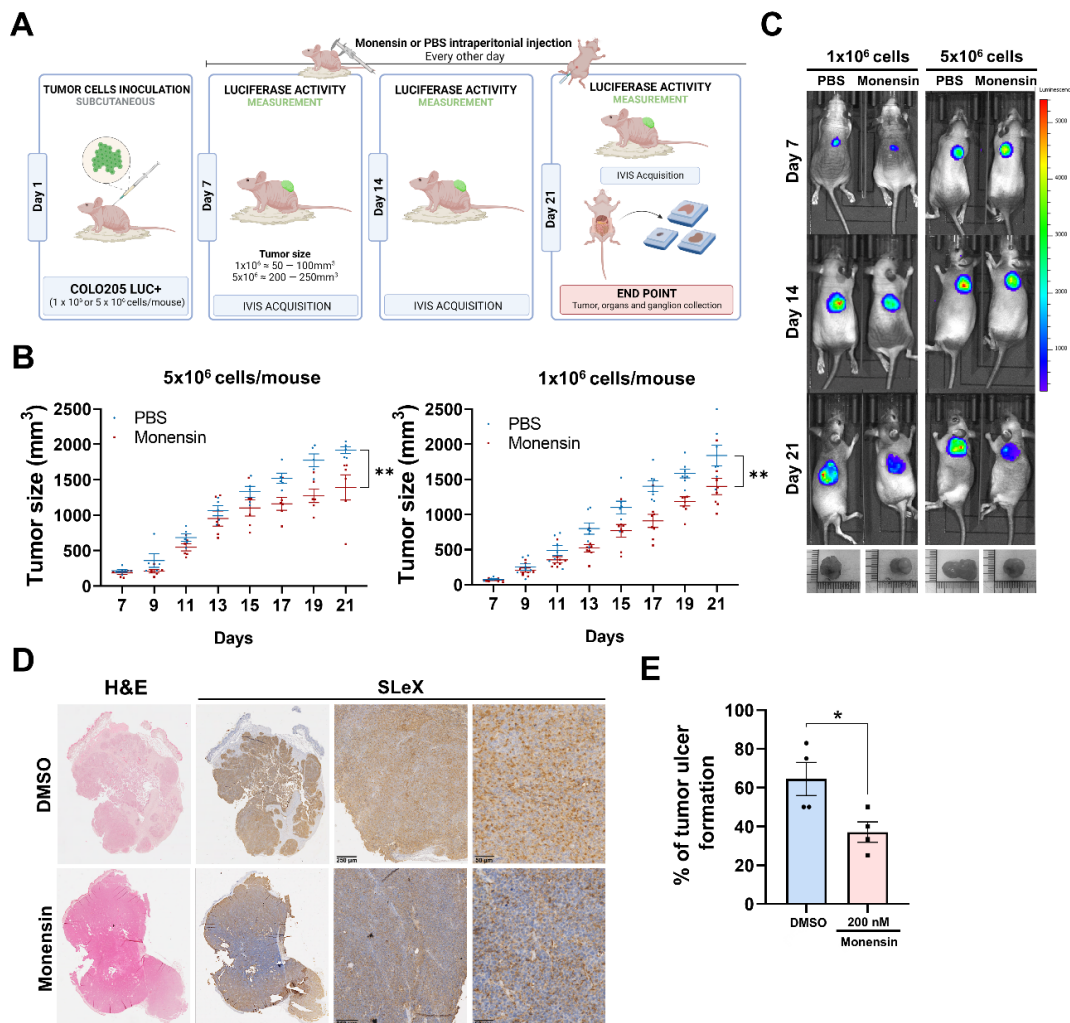


Figure 4: *In vivo* biological effect of monensin using a cell line xenograft mouse model. **A)** Schematic representation of the mice experiment. **B)** Tumor growth rate showing the reduced tumor burden in monensin-treated mice along the time. **C)** Representative images of *in vivo* tumor growth, by measuring luciferase activity throughout time, showing the reduced tumor growth in monensin-treated mice. **D)** Tumor images of H&E staining and immunohistochemistry of SLeX expression in representative mice, demonstrating a reduction in SLeX immunostaining in tumors from monensin-treated mice. **E)** Quantification of tumor ulceration in mice, showing decreased number of ulcerative tumors in monensin-treated mice. Results are presented as mean $\pm$ SEM. * $P < 0.01$ vs. DMSO-treated group.

Monensin inhibits tumor cell proliferation in SLeX-expressing patient-derived organoids

Patient-derived organoids (PDOs) are recognized as important models for studying molecular pathways underlying carcinogenesis and therapeutic resistance, but also for conducting drug screening experiments [18-20]. We assessed monensin's therapeutic efficacy in four distinct GC-derived SLeX-positive PDOs representing papillary/ intestinal, poorly cohesive and mixed carcinoma subtypes, as well as in two organoids derived from normal mucosa adjacent to tumor (models established in the group, unpublished data). Monensin's IC₅₀ in PDOs was determined in a dose-response manner, using concentrations ranging from 0 to 400 nM. The results demonstrated a heterogeneous *in vitro* response to monensin, with normal derived organoids presenting an IC₅₀ of 140 nM, and GC-derived PDOs an IC₅₀ of 50 nM or resistant (Fig 5A and Fig S5A). Additionally, immunofluorescence analysis revealed the capacity of monensin to impair SLeX biosynthesis, as shown by its reduced expression at cell surface of treated PDOs (Fig 5B).

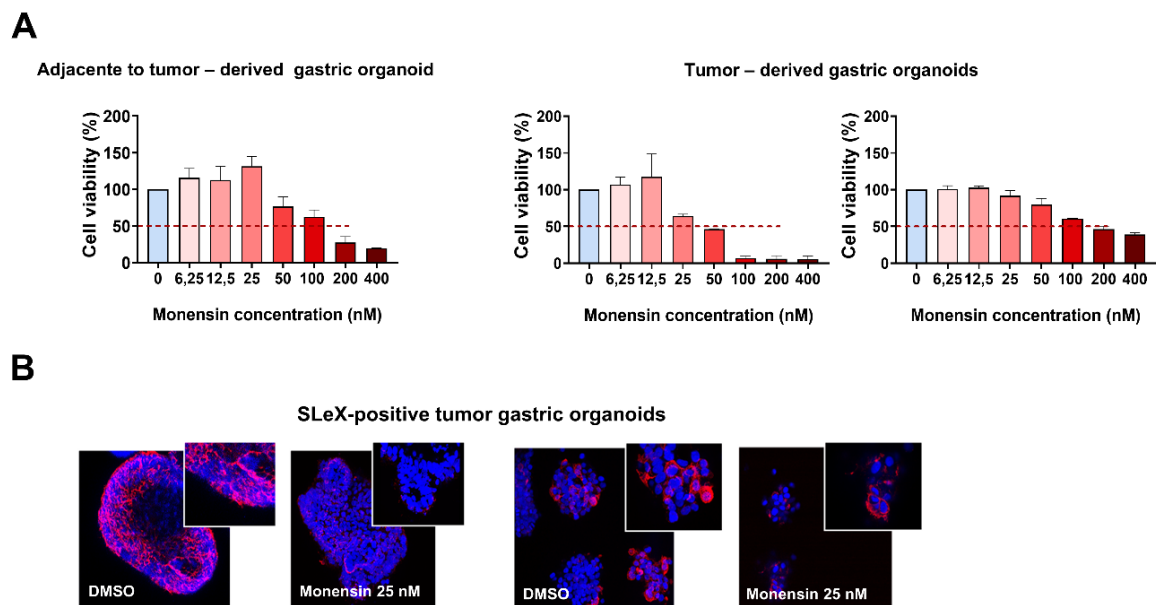


Figure 5: Biological effect of monensin in a pre-clinical organoid model. **A)** Viability assay of normal mucosa adjacent to tumor organoid (left) and two patient-derived GC organoids (right) after monensin treatment,

showing differences in sensitivity to treatment in the different models. **B)** Immunofluorescence analysis of SLeX expression in SLeX-positive GC PDOs after monensin treatment showing the reduction of SLeX.

To further validate monensin therapeutic activity under physiological conditions, we used a Patient-Derived Organoid Xenograft (PDOX) mouse model. PDOs were subcutaneously implanted in mice dorsal area, and when tumors reached approximately 100mm³ in size, were treated with monensin via IP administration (Fig 6A). Importantly, the four PDOX models preserved the histoarchitecture and SLeX expression pattern of PDOs (Fig 6B). Notably, none of the normal mucosa adjacent to tumor-derived organoids, as well as SLeX-negative GC PDOs successfully developed in mice, limiting monensin testing to SLeX-positive GC-PDOXs. Results showed a significant impact of monensin on tumor growth in 3 out of the 4 tested PDOXs, with a more pronounced effect observed in organoids expressing high levels of SLeX compared to those with moderate expression (Fig 6C). In the case of PDOX4, although no statistically significant difference in tumor size over time was observed, gross histological examination revealed a substantial necrotic area within monensin-treated PDOX4 compared to PBS control PDOX (Fig S5B). Neither monensin-treated mice nor control mice displayed signs of physiological or behavioral distress, demonstrating no significant side effects of monensin and therefore human endpoints were not considered along the experiment.

Histological examination of the tumors collected from the PDOX mice revealed a reduction in papillary-like structures in monensin-treated cases derived from papillary carcinomas (Fig 6D and Fig S5B and C). Additionally, the observed tumor bed of monensin-treated PDOXs is rich in necrotic areas and infiltrated immune cells, characteristic of tissue death. Furthermore, an impact on the protein secretory pathway was evident, with tumor cells exhibiting a cytoplasmic diffusely stained for SLeX in monensin-treated mice, in contrast to the clear membrane staining in PBS-treated mice (Fig 6D).

A combination of monensin with a largely used chemotherapy drug, 5-fluorouracil (5-FU), was further tested in the PDOX mouse model. Generally, 5-FU alone could not reduce tumor growth as effectively as monensin. However, the combination of both drugs resulted in an improved reduction of tumor size in PDOX 2 and 4. Especially in PDOX 4, where monensin treatment did not impact measured tumor size, the combination of monensin and 5-FU resulted in a significant reduction in tumor volume. On the other hand, the combination of drugs did not improve the reduction of tumor growth in PDOX 1 and 3, compared to monensin treatment alone (Fig 6E).

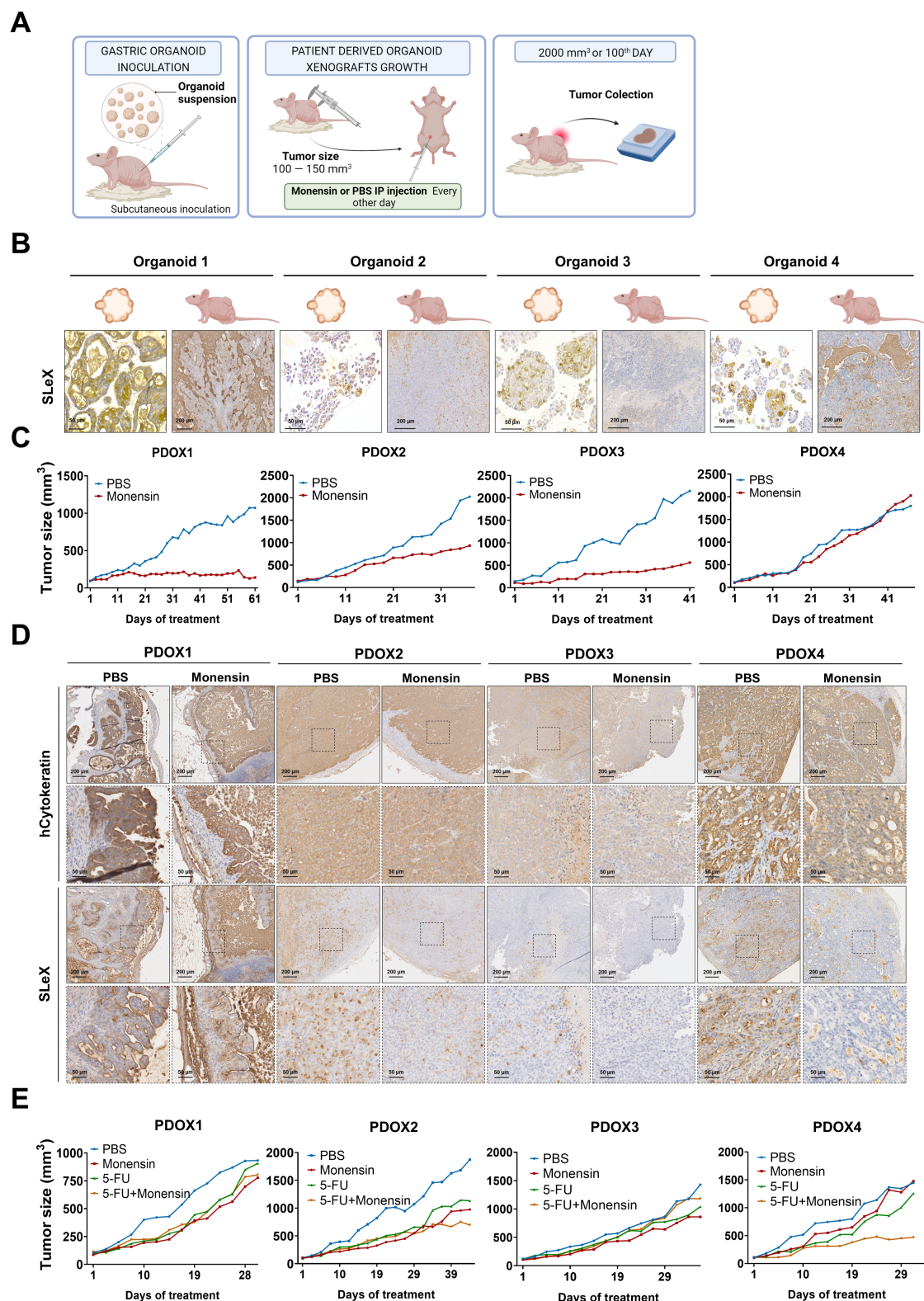


Figure 6: Biological effect of monensin in a PDO xenograft (PDOX) mouse model. A) Schematic representation of the experimental design followed to establish a patient-derived organoid xenograft (PDOX) mouse model. **B)** Histology (H&E) and SLeX expression of tumor-derived GC organoids and their respective PDOX tumor tissue collected from mice, showing a preserved differentiation status, histoarchitecture, and SLeX expression pattern. **C)** Tumor growth rate of GC PDOXs showing the reduction of tumor size in monensin-treated mice, compared to PBS-treated mice. **D)** Histological characterization of GC PDOXs tumors, showing

a preserved tumor histoarchitecture (human cytokeratin staining) and a reduction of SLeX immunostaining in tumors from monensin-treated mice. **E)** Tumor growth rate of GC PDOXs showing a higher reduction of tumor size in PDOX1 and PDOX3 upon treatment with monensin alone when compared to other tested conditions, or a synergistic effect of monensin with 5-FU in PDOX 2 and PDOX4 when compared to other tested conditions.

Discussion

CRC and GC rank at the top deadliest cancers, primarily due to their diagnosis at advanced stages where metastasis already occurred [2]. Indeed, recent statistics underscore CRC as top leading cancer in men and second in women under the age of 50, emphasizing the urgent need for new and effective treatment options [21]. SLeX plays an undeniably central role within the molecular cascade underlying the metastization process of both CRC and GC [as reviewed in 9]. Herein, we designed a cellular HTS-based assay to identify inhibitors of the biosynthetic pathway of this aberrantly expressed glycan that could serve as therapeutic agents against CRC and GC. Remarkably, we successfully pinpointed monensin as the most promising inhibitor of SLeX in both CRC and CG cells. Monensin, a polyether ionophore, is a fermentation product derived from *Streptomyces cinnamonensis*. Initially discovered in 1967 by Agtarap *et al.* [22], monensin finds widespread use in veterinary practice for addressing bacterial, fungal, and parasitic infections, particularly in poultry and cattle production.

Drug repurposing strategies emerge as cost-effective and promising avenues for discovering novel small molecule-based therapies [23]. Despite the inherent challenges and limitations associated with drug repurposing, several anticancer drugs repurposed from existing medications are currently advancing through clinical trials, with some already obtaining approval [24]. Monensin, recognized as a promising repurposing cancer drug, has garnered attention in previous HTS-based studies and it has been extensively explored across various tumor types [25-37]. Despite the studies that support its use as a promising drug for cancer treatment, no clinical trials have been conducted, and concerns regarding its toxicity in humans persist. Toxicity reports typically involve the ingestion of uncontrolled dosages of monensin formulas used for the poultry or cattle industry [33,38-41]. Thus, it is important the control of monensin dosage, formulation, and administration protocols to consider its clinical use.

In this study, we showed the capacity of monensin to inhibit SLeX, through the disruption of the well-coordinated glycosylation process, mainly O-glycosylation with a concomitant increased expression of the truncated O-glycan Tn antigen. We mainly attribute these glycan changes to the observed morphological and functional alterations in the Golgi apparatus structure, crucial for protein O-glycosylation and terminal N-glycosylation, as shown by TEM analysis and the use of ERGIC and Golgi markers. In fact,

previous studies have highlighted the impact of monensin on glycosylation. These studies reported undersulfation of glycosaminoglycans and alterations in both *N*- and *O*-linked glycans [42]. Nonetheless, there are very few reports describing a direct link between monensin and altered glycan biosynthesis, with the main evidence focusing on alterations to the Golgi morphology and function [43,44].

Given its characteristics as an ionophore, monensin deregulates cation influxes, specifically Na^+ and Ca^{2+} , within the Golgi compartment [45]. This disruption promotes, as observed by TEM analysis, a noticeably swollen phenotype, compromising the Golgi's function and consequently impacting protein glycosylation, processing, and secretion [44]. In fact, the present work revealed an altered expression in genes related to ER stress, protein folding and secretion, and cell adhesion processes, as shown by transcriptomic analysis. Our findings showed that there was an impairment of SLeX protein carriers at the cell membrane, as well as the secreted forms, alongside abnormal expression of cell-adhesion molecules such as E-cadherin in treated cells. Indeed, monensin has been used as a standard reagent in the inhibition of protein transport to the membrane [24] and cytokine secretion [46,47], corroborating the observed effects. Additionally, monensin has been described as an inducer of oxidative stress, hindering cell growth by inducing G1-phase cell cycle arrest, ultimately resulting in cancer cell apoptosis [28,32]. The oxidative stress generated has been reported to alter mitochondrial function [25,37], a phenomenon also observed in GC cells used in our study.

In this study, we have also observed an increased *in vitro* sensitivity of SLeX-positive cancer cells to monensin. Specifically, we showed that, from six GC and CRC cell lines, the tested monensin concentrations impacted only the two SLeX-positive cell lines, potentially narrowing down its effect on cells expressing this particular glycan structure. It was described that monensin forms stable complexes with lipid membranes acting as cation carriers, enabling rapid traversal through cell membranes [48]. Building on this, we hypothesize that the anionic charge conferred by sialic acids at the cell surface of SLeX-positive cancer cells potentially facilitates monensin's uptake and intracellular transport, rendering SLeX-positive cancer cells more sensitive to treatment. This hypothesis aligns not only with the differences in the metabolic responses of monensin-treated SLeX-positive and SLeX-negative cells but also with an increased immunodetection of clathrin, a fundamental protein in intracellular vesicle formation responsible for extracellular material uptake into cells [49,50]. Consistent with this evidence, Vanneste *et al.* conducted a screening of 23 human cancer cell lines to assess their sensitivity to monensin. Their findings revealed 12 cell lines responsive and 11 cell lines resistant to monensin, from which the CRC cells HT-29 and HCT-116 exhibit sensitivity and resistance to monensin, respectively [32]. Notably, HT-29 is a SLeX-positive cell line, whereas HCT-116 is a SLeX-

negative cell line [10], underscoring the potential of SLeX in influencing the sensitivity of cancer cells to monensin treatment.

SLeX-positive tumors are inherently associated with more aggressive and invasive cancer behaviors [6,7,10]. In the present study, we pointed out monensin's efficacy in inhibiting SLeX expression, suppressing cancer cell motility and invasion in cell-based assays, and leading to a sustained reduction in tumor growth across various *in vivo* models, without inducing any toxic effects in the mice. This aligns with a recent report highlighting monensin's specificity in targeting hepatocellular carcinoma allografts without adversely affecting normal hepatic and non-hepatic tissues. This selectivity is attributed to the energetic incapacity of hepatocellular carcinoma cells to compensate for and survive the Na⁺ load induced by monensin [51].

Combination therapy is a key approach in cancer treatment as targeting multiple pathways can help reduce drug resistance [52]. In this context, we tested a combination of monensin with 5-FU, a widely used chemotherapeutic drug in CRC and GC cancer treatment [53,54]. This combination led to a significant reduction in tumor growth compared to monensin or 5-FU alone in half of the tested PDOX models. The variation in efficacy and sensitivity to monensin or 5-FU observed across the different PDOX models reflects the molecular heterogeneity of tumors from the different patients. Each PDO is derived from a tumor that presented unique characteristics such as individual genetic background, cancer genetic alterations or histological subtype, as well as expression of cancer markers such as SLeX. However, in cases such as PDOX 4, where neither monensin nor 5-FU alone could reduce tumor volume, the combination significantly improved the efficacy of both drugs. Furthermore, previous studies have already shown the synergistic action of monensin with other chemotherapy drugs [55,56], or other cancer therapies [33,57,58].

Overall, our study contributes to the evolving landscape of cancer drug discovery by demonstrating the remarkable capacity of monensin to target SLeX-positive CRC and GC cells. Beyond its impact on glycosylation pathways and SLeX expression, monensin exerts an influence on associated malignant properties, notably impeding tumor growth and invasion. This not only underscores the versatility of monensin but also positions it as a potential therapeutic candidate for cancer treatment.

Conclusion

The identification of novel and effective cancer treatment options is paramount in cancer research. Nevertheless, progress is hindered by the currently available discovery approaches that often yield ineffective drugs in clinical trials. Recent studies have explored the potential of repurposing existing drugs, demonstrating its huge potential for the development of anticancer drugs [59].

This work acknowledges the importance of targeting cancer-specific glycans as a new source of cancer drugs and further addresses how SLeX inhibition by monensin can impair the malignant behavior of cancer cells. Overall, this study highlights the significant potential of monensin to disrupt SLeX biosynthesis at the Golgi within cancer cells and impair tumor growth and motility capacities positioning monensin as an anticancer agent for CRC and GC.

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Contributions

CG and CR supervised the study. CG designed the experiments. DC, AP, CG and AFM performed and analyzed the HTS. LSF and FP establish the organoid models. AFC, ES, AT, IFR, SL, JG, CG and MTP performed the *in vivo* experiments. VF and LP analyzed the RNASeq data. AFC, ES, DC, LSF, AT, RA, HOD, MP, AT and CG performed the

remaining experiments and analyzed the data. RB, FCG, FS and JB collected and provided patient gastric tissues. FC evaluated the PDOX histology. CG and AFC wrote the original paper. All authors critically revised the paper.

Ethical Statement

All procedures concerning the inclusion of patients were approved by the Centro Hospitalar Universitário de São João Ethics Committee (CES 223/2021). Also, all sample collection procedures were conducted following the patients' informed consent.

Conflicts of Interest

The authors declare no conflict of interest.

References

- [1] Tredan O, Galmarini CM, Patel K, et al. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst.* 2007 Oct 3;99(19):1441-54.
- [2] Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2021 May;71(3):209-249.
- [3] Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer.* 2015 Sep;15(9):540-55.
- [4] Mereiter S, Balmana M, Campos D, et al. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? *Cancer Cell.* 2019 Jul 8;36(1):6-16.
- [5] Amado M, Carneiro F, Seixas M, et al. Dimeric sialyl-Le(x) expression in gastric carcinoma correlates with venous invasion and poor outcome. *Gastroenterology.* 1998 Mar;114(3):462-70.
- [6] Gomes C, Almeida A, Barreira A, et al. Carcinoembryonic antigen carrying SLe(X) as a new biomarker of more aggressive gastric carcinomas. *Theranostics.* 2019 Oct;9(24):7431-7446.
- [7] Gomes C, Osorio H, Pinto MT, et al. Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS One.* 2013 Jun;8(6):e66737.
- [8] Varki A. Sialic acids in human health and disease. *Trends Mol Med.* 2008 Aug;14(8):351-60.
- [9] Trinchera M, Aronica A, Dall'Olio F. Selectin Ligands Sialyl-Lewis a and Sialyl-Lewis x in Gastrointestinal Cancers. *Biology (Basel).* 2017 Feb 23;6(1).

- [10] Costa AF, Senra E, Faria-Ramos I, et al. ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and associates with cancer cell motility. *Glycoconj J*. 2023 Aug;40(4):421-433.
- [11] Costa AF, Campos D, Reis CA, et al. Targeting Glycosylation: A New Road for Cancer Drug Discovery. *Trends in Cancer*. 2020 Set;6(9):757-766.
- [12] Gloster TM, Vocadlo DJ. Developing inhibitors of glycan processing enzymes as tools for enabling glycobiology. *Nat Chem Biol*. 2012 Jul 18;8(8):683-94.
- [13] Magnani JL, Ernst B. Glycomimetic drugs--a new source of therapeutic opportunities. *Discov Med*. 2009 Dec;8(43):247-52.
- [14] Sorensen DM, Bull C, Madsen TD, et al. Identification of global inhibitors of cellular glycosylation. *Nat Commun*. 2023 Feb 20;14(1):948.
- [15] Berg S, Kutra D, Kroeger T, et al. ilastik: interactive machine learning for (bio)image analysis. *Nat Methods*. 2019 Dec;16(12):1226-1232.
- [16] McQuin C, Goodman A, Chernyshev V, et al. CellProfiler 3.0: Next-generation image processing for biology. *PLoS Biol*. 2018 Jul;16(7):e2005970.
- [17] Leupold JH, Patil N, Allgayer H. The Chicken Egg Chorioallantoic Membrane (CAM) Model as an In Vivo Method for the Investigation of the Invasion and Metastasis Cascade of Malignant Tumor Cells. *Methods Mol Biol*. 2021 Mar;2294:17-26.
- [18] Broutier L, Mastrogiovanni G, Verstegen MM, et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nat Med*. 2017 Dec;23(12):1424-1435.
- [19] Crespo M, Vilar E, Tsai SY, et al. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. *Nat Med*. 2017 Jul;23(7):878-884.
- [20] Chakradhar S. Put to the test: Organoid-based testing becomes a clinical tool. *Nat Med*. 2017 Jul 11;23(7):796-799.
- [21] Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin*. 2024 Jan-Feb;74(1):12-49.
- [22] Agtarap A, Chamberlin JW, Pinkerton M, et al. The structure of monensic acid, a new biologically active compound. *J Am Chem Soc*. 1967 Oct 25;89(22):5737-9.
- [23] Bhattarai D, Singh S, Jang Y, et al. An Insight into Drug Repositioning for the Development of Novel Anti-Cancer Drugs. *Curr Top Med Chem*. 2016 Feb;16(19):2156-68.
- [24] Schein CH. Repurposing approved drugs for cancer therapy. *Br Med Bull*. 2021 Mar 25;137(1):13-27.

- [25] Charvat RA, Arrizabalaga G. Oxidative stress generated during monensin treatment contributes to altered *Toxoplasma gondii* mitochondrial function. *Sci Rep*. 2016 Mar 15;6:22997.
- [26] Deng Y, Zhang J, Wang Z, et al. Antibiotic monensin synergizes with EGFR inhibitors and oxaliplatin to suppress the proliferation of human ovarian cancer cells. *Scientific Reports*. 2015 Dec;5(1):17523.
- [27] Iljin K, Ketola K, Vainio P, et al. High-Throughput Cell-Based Screening of 4910 Known Drugs and Drug-like Small Molecules Identifies Disulfiram as an Inhibitor of Prostate Cancer Cell Growth. *Clinical Cancer Research*. 2009 Oct;15(19):6070-6078.
- [28] Ketola K, Vainio P, Fey V, et al. Monensin Is a Potent Inducer of Oxidative Stress and Inhibitor of Androgen Signaling Leading to Apoptosis in Prostate Cancer Cells. *Molecular Cancer Therapeutics*. 2010 Dec;9(12):3175-3185.
- [29] Mottola-Hartshorn C, Lorenzoni P, Ceccarini C. Biosynthesis and glycosylation of the carcinoembryonic antigen. *Biochem J*. 1988 Nov 1;255(3):943-8.
- [30] Secme M, Kocoglu SS. Investigation of the TLR4 and IRF3 signaling pathway-mediated effects of monensin in colorectal cancer cells. *Med Oncol*. 2023 May 23;40(7):187.
- [31] Tumova L, Pombinho AR, Vojtechova M, et al. Monensin inhibits canonical Wnt signaling in human colorectal cancer cells and suppresses tumor growth in multiple intestinal neoplasia mice. *Mol Cancer Ther*. 2014 Apr;13(4):812-22.
- [32] Vanneste M, Huang Q, Li M, et al. High content screening identifies monensin as an EMT-selective cytotoxic compound. *Sci Rep*. 2019 Feb 4;9(1):1200.
- [33] Wang X, Wu X, Zhang Z, et al. Monensin inhibits cell proliferation and tumor growth of chemo-resistant pancreatic cancer cells by targeting the EGFR signaling pathway. *Scientific Reports*. 2018 Dec;8(1).
- [34] Xin H, Li J, Zhang H, et al. Monensin may inhibit melanoma by regulating the selection between differentiation and stemness of melanoma stem cells. *PeerJ*. 2019 Jul;7:e7354.
- [35] Zhou Y, Deng Y, Wang J, et al. Effect of antibiotic monensin on cell proliferation and IGF1R signaling pathway in human colorectal cancer cells. *Ann Med*. 2023 Dec;55(1):954-964.
- [36] Padua D, Barros R, Amaral AL, et al. A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin. *Cancers (Basel)*. 2020 Feb 20;12(2).
- [37] Mollenhauer HH, Rowe LD, Witzel DA. Effect of monensin on the morphology of mitochondria in rodent and equine striated muscle. *Vet Hum Toxicol*. 1984 Feb;26(1):15-9.

- [38] Caldeira C, Neves WS, Cury PM, et al. Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am J Kidney Dis.* 2001 Nov;38(5):1108-12.
- [39] Kouyoumdjian JA, Morita MP, Sato AK, et al. Fatal rhabdomyolysis after acute sodium monensin (Rumensin) toxicity: case report. *Arq Neuropsiquiatr.* 2001 Sep;59(3-A):596-8.
- [40] Zhang Z, Cui S, Zhang J. Rhabdomyolysis and hepatotoxicity following accidental monensin ingestion: A report of two cases. *Toxicol Ind Health.* 2021 Jan;37(1):34-37.
- [41] Blain M, Garrard A, Poppenga R, et al. Survival After Severe Rhabdomyolysis Following Monensin Ingestion. *J Med Toxicol.* 2017 Sep;13(3):259-262.
- [42] Yanagishita M, Hascall VC. Effects of monensin on the synthesis, transport, and intracellular degradation of proteoglycans in rat ovarian granulosa cells in culture. *J Biol Chem.* 1985 May 10;260(9):5445-55.
- [43] Mollenhauer HH, Morre DJ, Rowe LD. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim Biophys Acta.* 1990 May 7;1031(2):225-46.
- [44] Marjanovic M, Mikecin Drazic AM, Mioc M, et al. Salinomycin disturbs Golgi function and specifically affects cells in epithelial-to-mesenchymal transition. *J Cell Sci.* 2023 Sep 1;136(17).
- [45] Tsuchida K, Hirose H, Ozawa S, et al. Monensin-Induced Increase in Intracellular Na⁺ Induces Changes in Na⁺ and Ca²⁺ Currents and Regulates Na⁺-K⁺ and Na⁺-Ca²⁺ Transport in Cardiomyocytes. *Pharmacology.* 2021 Oct;106(1-2):91-105.
- [46] Kovacs SB, Oh C, Aachoui Y, et al. Evaluating cytokine production by flow cytometry using brefeldin A in mice. *STAR Protoc.* 2021 Mar 19;2(1):100244.
- [47] Lamoreaux L, Roederer M, Koup R. Intracellular cytokine optimization and standard operating procedure. *Nat Protoc.* 2006 Nov;1(3):1507-16.
- [48] Antonenko YN, Rokitskaya TI, Huczynski A. Electrogenic and nonelectrogenic ion fluxes across lipid and mitochondrial membranes mediated by monensin and monensin ethyl ester. *Biochim Biophys Acta.* 2015 Apr;1848(4):995-1004.
- [49] Chen CL, Hou WH, Liu IH, et al. Inhibitors of clathrin-dependent endocytosis enhance TGFβ signaling and responses. *J Cell Sci.* 2009 Jun 1;122(Pt 11):1863-71.
- [50] McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol.* 2011 Jul 22;12(8):517-33.
- [51] Clemente N, Baroni S, Fiorilla S, et al. Boosting intracellular sodium selectively kills hepatocarcinoma cells and induces hepatocellular carcinoma tumor shrinkage in mice. *Commun Biol.* 2023 May 29;6(1):574.

- [52] Bayat Mokhtari R, Homayouni TS, Baluch N, et al. Combination therapy in combating cancer. *Oncotarget*. 2017 Jun 6;8(23):38022-38043.
- [53] Sitarz R, Skierucha M, Mielko J, et al. Gastric cancer: epidemiology, prevention, classification, and treatment. *Cancer Manag Res*. 2018 Feb;10:239-248.
- [54] Adebayo AS, Agbaje K, Adesina SK, et al. Colorectal Cancer: Disease Process, Current Treatment Options, and Future Perspectives. *Pharmaceutics*. 2023 Nov 12;15(11).
- [55] Shaik MS, Chatterjee A, Singh M. Effects of monensin liposomes on the cytotoxicity, apoptosis and expression of multidrug resistance genes in doxorubicin-resistant human breast tumour (MCF-7/dox) cell-line. *J Pharm Pharmacol*. 2004 Jul;56(7):899-907.
- [56] Zeng C, Long M, Lu Y. Monensin synergizes with chemotherapy in uveal melanoma through suppressing RhoA. *Immunopharmacol Immunotoxicol*. 2023 Feb;45(1):35-42.
- [57] Dayekh K, Johnson-Obaseki S, Corsten M, et al. Monensin inhibits epidermal growth factor receptor trafficking and activation: synergistic cytotoxicity in combination with EGFR inhibitors. *Mol Cancer Ther*. 2014 Nov;13(11):2559-71.
- [58] Ochi K, Suzawa K, Tomida S, et al. Overcoming epithelial-mesenchymal transition-mediated drug resistance with monensin-based combined therapy in non-small cell lung cancer. *Biochem Biophys Res Commun*. 2020 Aug 27;529(3):760-765.
- [59] Pushpakom S, Iorio F, Eyers PA, et al. Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discov*. 2019 Jan;18(1):41-58.

Supplementary material for:

Monensin as a potential drug for treatment of SLeX-positive tumors

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→ **Supplementary Figure 1-5**

Diverset Plate 1

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
A	512	02	03	04	05	06	07	08	4038	1543	3253	308	2510	368	4045	115	4372	4288	3901	58	5606	2918	2743	40	885	190
B	15	204	321	275	355	3054	248	2630	355	840	390	2518	365	3723	278	3149	372	382	273	272	392	306	287	131	131	
C	246	481	1135	188	2746	2746	3525	2007	1286	2531	3096	3555	4019	4348	4441	5	3863	3442	3441	3440	3978	4135	3467	3054	20	
D	38	2476	308	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	2746	
E	1150	396	1004	409	2378	2874	3196	3247	3597	3602	2526	2101	3716	3861	3406	3608	3416	4214	2965	426	1019	427	3275	167	167	
F	395	304	167	304	167	304	167	304	167	304	167	304	167	304	167	304	167	304	167	304	167	304	167	304	167	
G	52	38	396	1654	1467	1583	1232	368	2528	2594	1845	266	2118	2291	2535	2275	2556	3095	2678	2487	2504	3426	2490	46	111	
H	103	648	968	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	1580	1291	
I	2265	103	103	1683	188	2022	328	1022	328	1022	328	1022	328	1022	328	1022	328	1022	328	1022	328	1022	328	1022	328	
J	390	375	375	375	233	2333	2504	2060	2314	2666	2740	2447	1020	3219	3805	3480	3319	3899	272	4388	3696	523	423	288	55	
K	923	145	110	107	1277	164	308	343	308	343	308	343	308	343	308	343	308	343	308	343	308	343	308	343	308	
L	6	156	214	336	336	930	2022	2309	2422	2191	355	1152	112	462	1906	2745	2790	479	2422	138	2678	479	281	248	55	
M	19	149	189	38	1423	234	212	238	2127	388	2828	388	3013	378	2474	2871	2890	378	273	2456	2973	438	446	231	231	
N	72	97	161	177	178	189	238	238	1112	318	1654	260	2460	252	327	250	348	2005	370	264	295	283	298	298	298	
O	946	19	809	148	1568	164	1548	158	1825	114	279	2160	279	2360	282	2384	282	3023	289	2405	279	264	217	2500	134	
P	5	63	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
A	433	637	407	312	260	715	363	420	293	661	270	738	321	772	290	325	294	244	777	240	361	281	585	620	180	
B	635	272	724	700	718	345	278	355	661	354	684	400	648	120	647	300	665	643	622	633	638	627	293	129	129	
C	1338	476	329	605	266	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	272	
D	550	315	405	277	453	305	302	304	289	311	281	308	701	271	251	229	205	290	435	260	260	250	646	178	178	
E	761	764	313	632	366	296	300	314	289	281	389	371	289	295	304	277	302	233	309	692	431	638	111	126	126	
F	464	761	730	737	669	728	315	310	278	319	302	315	293	282	587	593	549	264	573	552	674	275	684	125	125	
G	661	423	390	313	374	379	176	658	323	309	348	658	345	324	300	302	320	352	320	324	319	367	327	126	126	
H	428	482	427	500	383	427	444	331	301	355	353	268	321	387	304	266	310	510	289	299	315	303	632	129	129	
I	239	587	496	662	330	608	446	808	111	308	302	262	292	299	279	294	535	271	271	317	274	313	610	129	129	
J	820	743	698	688	364	325	379	377	796	306	304	642	412	299	284	394	279	295	798	286	296	617	140	688	127	
K	430	801	737	705	451	676	846	346	646	377	367	317	380	380	439	320	339	321	320	341	321	659	417	119	119	
L	400	604	676	676	735	651	662	305	344	330	312	317	615	344	302	290	541	144	282	304	607	296	642	81	81	
M	439	838	647	858	442	719	372	688	357	695	340	667	687	687	663	365	363	355	652	350	413	368	635	627	118	118
N	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	761	
O	339	625	410	782	359	669	612	618	383	643	345	345	667	276	567	305	271	306	616	279	534	248	577	628	134	134
P	238	667	666	443	585	618	663	607	679	690	504	279	544	245	514	253	293	236	281	198	507	431	449	111	111	

Diverset Plate 2

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24			
A	301	1144	1382	2453	3710	4810	3328	3798	2209	2007	1540	2738	1871	2613	2517	2591	3413	4407	4669	4678	4697	5182	4391	4039	3882	3880	2485
B	2129	3850	1784	3428	4945	2970	4255	3710	4592	3627	4638	3795	3590	4683	3607	4669	4678	4697	5182	4391	4039	3882	3880	2485	2485	2485	2485
C	1737	607	388	1145	2710	2188	2175	2412	3938	4524	4021	3587	3795	4524	4021	3587	3795	4524	4021	3587	3795	4524	4021	3587	3795	4524	4021
D	1346	905	1483	1682	2421	3357	2820	2560	2505	3025	3025	2673	2673	3025	3025	2673	3025	3025	2673	3025	3025	2673	3025	3025	2673	3025	3025
E	1875	369	781	2833	3132	3052	3831	3014	3613	3873	3690	4138	4137	4122	4536	3690	3848	4011	3695	4536	3690	3848	4011	3695	4536	3690	3848
F	2817	2079	1883	1665	3504	4063	3504	3441	4377	3863	4450	4078	4078	4450	4078	4078	4450	4078	4078	4450	4078	4078	4450	4078	4078	4450	4078
G	9	5	45	474	765	650	1130	861	1328	2000	1089	1840	1581	1890	1591	1379	168	2141	2181	1547	2402	2433	1623	942	942	942	942
H	379	115	795	795	1486	608	1455	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643
I	1377	470	325	508	1934	1794	793	1040	1627	1773	2652	2047	262	1819	2324	2090	2165	1238	2238	1238	2637	2464	2883	3075	2343	2343	2343
J	1590	447	193	1363	1378	1486	1278	2682	1981	2191	2028	1566	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564
K	1039	133	428	2866	1195	3068	3154	2296	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603
L	1456	754	934	788	1131	1108	1008	1032	1545	1506	1388	1666	1429	1737	1736	1796	2007	2235	1796	2007	2235	1796	2007	2235	1796	2007	2235
M	417	671	180	514	323	318	306	258	266	240	260	278	249	309	277	302	309	280	307	355	253	295	286	117	117	117	117
N	3067	950	344	1275	1134	1056	1334	2986	1535	895	1898	2447	1287	1161	1572	2442	1249	1381	389	1099	2048	1125	1814	46	46	46	46
O	1387	957	1105	2056	1561	1652	1795	716	974	2950	1522	3085	2986	2691	3074	1052	2725	363	2095	3038	1969	2649	1466	1971	1971	1971	1971

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24			
A	467	311	493	771	221	240	248	257	311	327	370	246	355	312	346	336	258	244	276	231	290	282	275	117	117	117	117
B	381	248	412	230	243	251	298	242	247	269	245	241	246	279	214	260	258	251	243	253	262	267	252	292	115	115	115
C	287	417	638	638	327	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	
D	490	521	479	390	354	385	327	327	343	311	318	335	342	448	292	296	283	343	303	295	310	288	175	114	114	114	
E	417	671	180	514	323	318	306	258	266	240	260	278	249	309	277	302	309	280	307	355	253	295	286	117	117	117	
F	3067	950	344	1275	1134	1056	1334	2986	1535	895	1898	2447	1287	1161	1572	2442	1249	1381	389	1099	2048	1125	1814	46	46	46	
G	1387	957	1105	2056	1561	1652	1795	716	974	2950	1522	3085	2986	2691	3074	1052	2725	363	2095	3038	1969	2649	1466	1971	1971	1971	
H	9	5	45	474	765	650	1130	861	1328	2000	1089	1840	1581	1890	1591	1379	168	2141	2181	1547	2402	2433	1623	942	942	942	
I	379	115	795	795	1486	608	1455	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	
J	1377	470	325	508	1934	1794	793	1040	1627	1773	2652	2047	262	1819	2324	2090	2165	1238	2238	1238	2637	2464	2883	3075	2343	2343	
K	1590	447	193	1363	1378	1486	1278	2682	1981	2191	2028	1566	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	
L	1039	133	428	2866	1195	3068	3154	2296	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	
M	1456	754	934	788	1131	1108	1008	1032	1545	1506	1388	1666	1429	1737	1736	1796	2007	2235	1796	2007	2235	1796	2007	2235	1796	2007	
N	417	671	180	514	323	318	306	258	266	240	260	278	249	309	277	302	309	280	307	355	253	295	286	117	117	117	
O	3067	950	344	1275	1134	1056	1334	2986	1535	895	1898	2447	1287	1161	1572	2442	1249	1381	389	1099	2048	1125	1814	46	46	46	
P	1387	957	1105	2056	1561	1652	1795	716	974	2950	1522	3085	2986	2691	3074	1052	2725	363	2095	3038	1969	2649	1466	1971	1971	1971	

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
A	467	311	493	771	221	240	248	257	311	327	370	246	355	312	346	336	258	244	276	231	290	282	275	117	117	117
B	381	248	412	230	243	251	298	242	247	269	245	241	246	279	214	260	258	251	243	253	262	267	252	292	115	115
C	287	417	638	638	327	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	
D	490	521	479	390	354	385	327	327	343	311	318	335	342	448	292	296	283	343	303	295	310	288	175	114	114	
E	417	671	180	514	323	318	306	258	266	240	260	278	249	309	277	302	309	280	307	355	253	295	286	117	117	
F	3067	950	344	1275	1134	1056	1334	2986	1535	895	1898	2447	1287	1161	1572	2442	1249	1381	389	1099	2048	1125	1814	46	46	
G	1387	957	1105	2056	1561	1652	1795	716	974	2950	1522	3085	2986	2691	3074	1052	2725	363	2095	3038	1969	2649	1466	1971	1971	
H	9	5	45	474	765	650	1130	861	1328	2000	1089	1840	1581	1890	1591	1379	168	2141	2181	1547	2402	2433	1623	942	942	
I	379	115	795	795	1486	608	1455	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278	2643	1242	1278
J	1377	470	325	508	1934	1794	793	1040	1627	1773	2652	2047	262	1819	2324	2090	2165	1238	2238	1238	2637	2464	2883	3075	2343	2343
K	1590	447	193	1363	1378	1486	1278	2682	1981	2191	2028	1566	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	1564	
L	1039	133	428	2866	1195	3068	3154	2296	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	2603	
M	1456	754	934	788	1131	1108	1008	1032	1545	1506	1388	1666	1429	1737	1736	1796	2007	2235	1796	2007	2235	1796	2007	2235	1796	
N	417	671	180	514	323	318	306	258	266	240	260	278	249	309	277	302	309	280	307	355	253	295	286	117	117	
O	3067	950	344	1275	1134	1056	1334	2986	1535	895	1898	2447	1287	1161	1572	2442	1249	1381	389	1099	2048	1125	1814	46	46	
P	1387	957	1105	2056	1561	1652	1795	716	974	2950	1522	3085	2986	2691	3074	1052	2725	363	2095	3038	1969	2649	1466	1971	1971	

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
A	467	311	493	771	221	240	248	257	311	327	370	246	355	312	346	336	258	244	276	231	290	282	275	117	117	117
B	381	248	412	230	243	251	298	242	247	269	245	241	246	279	214	260	258	251	243	253	262	267	252	292	115	115
C	287	417	638	638	327	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	372	
D	490	521	479	390	354	38																				

Diverset Plate 5

Row\Col	Col																								
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	590	1057	2080	2798	1441	06	07	08	2190	3883	2021	27	173	138	2888	2516	879	1042	2041	1207	1950	241	1638	1887	2028
B	1210	1038	1118	1767	2686	1386	2111	2070	1827	3886	2676	1571	854	1867	2601	959	1247	1741	3168	2661	2758	2322	1148	1847	3079
C	1217	318	1005	1470	1666	2070	1333	1296	663	1887	1097	896	1881	1795	1888	1085	1714	1789	2600	2452	2492	1216	2941	2855	2841
D	1644	943	1118	1864	1700	1488	188	1448	2240	1198	1488	188	1448	2240	1198	1488	188	1448	2240	1198	1488	188	1448	2240	1198
E	1952	1457	1042	2103	2120	1581	1704	1154	1170	2183	1874	1361	1518	2287	2289	2274	2155	2009	1789	2274	2487	2394	2247	1984	
F	1243	1569	1714	220	2643	1176	1571	2158	1796	1566	121	1913	2055	2086	1709	1960	2061	2050	1858	2367	2415	1912	2171	949	
G	297	30	190	519	627	205	308	1129	993	977	1734	1214	1247	1582	2182	1208	674	2045	2770	2408	1730	2487	1888	1730	
H	458	197	356	658	1020	590	941	1274	732	522	1256	968	957	1075	1688	398	1117	1618	1625	2214	1713	1974	2462	1713	
I	427	496	485	878	951	814	814	1012	951	920	986	118	882	830	942	1169	1899	1482	1700	2104	2013	1514	2167	364	
J	390	1222	705	281	29	544	1091	741	653	991	381	883	957	1435	814	1482	1144	1273	1494	1531	1730	1172	2238	1895	
K	127	54	272	595	387	801	499	809	900	760	517	802	1481	1384	1159	2005	940	1170	1659	2161	2029	1967	1882	1948	
L	17	85	81	634	147	600	484	490	268	676	83	246	746	921	951	697	1051	977	843	1698	228	1079	1803	1480	
M	127	54	272	595	387	801	499	809	900	760	517	802	1481	1384	1159	2005	940	1170	1659	2161	2029	1967	1882	1948	
N	67	65	495	293	238	451	306	505	454	479	320	963	1480	1012	848	1419	1658	534	1562	1657	1462	1313	254	254	
O	114	128	188	181	273	245	283	146	234	1079	217	195	293	714	432	571	508	1169	358	468	1046	145	724	1351	477
P	460	197	356	658	1020	590	941	1274	732	522	1256	968	957	1075	1688	398	1117	1618	1625	2214	1713	1974	2462	1713	

DAP1
(Cell Count)

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	909	351	379	528	278	441	060	776	072	1888	567	384	472	1220	205	442	608	527	377	504	445	761	1668	480
B	189	55	403	428	458	395	465	408	458	458	458	458	710	465	458	458	458	458	458	458	458	458	458	458
C	398	860	562	548	554	558	768	571	774	513	506	565	356	462	704	541	697	419	336	303	420	470	433	
D	416	364	582	710	280	282	414	397	430	435	245	276	386	284	264	773	264	272	114	236	269	207	277	
E	368	511	378	511	427	441	557	368	511	378	511	378	511	378	511	378	511	378	511	378	511	378	511	
F	188	882	882	512	462	512	469	512	469	512	469	512	469	512	469	512	469	512	469	512	469	512	469	
G	178	1378	1378	536	536	489	786	445	638	833	518	544	776	600	456	817	805	589	548	467	552	507	808	449
H	708	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	704	
I	1174	572	572	992	3200	601	587	542	608	479	459	809	719	742	604	629	572	640	678	471	574	703	651	474
J	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	1384	
K	1461	1348	1026	891	1399	65	787	742	1088	058	1609	713	703	821	691	640	572	640	678	471	574	703	651	474
L	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	1552	
M	1020	1188	585	1214	965	741	1088	950	1335	960	1892	703	560	792	786	582	803	1043	631	493	541	473	503	
N	1020	1188	585	1214	965	741	1088	950	1335	960	1892	703	560	792	786	582	803	1043	631	493	541	473	503	
O	590	446	1092	855	685	454	1088	977	791	821	1392	830	567	1229	738	646	804	442	698	442	698	442	698	442

TEXAS RED

Row\Col	Col																							
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	901	351	379	278	741	448	776	471	348	557	344	472	1220	808	442	608	527	377	504	445	761	988	488	128
B	389	352	526	713	403	395	387	448	486	311	340	578	655	445	740	542	451	481	421	445	405	390	730	336
C	398	840	562	548	514	358	768	171	774	513	506	546	396	442	704	541	407	410	246	368	420	470	419	125
D	383	601	492	985	696	615	1176	422	487	696	443	549	456	601	384	433	374	366	407	404	395	340	739	121
E	436	348	512	340	280	282	314	397	430	245	276	386	284	274	234	273	264	272	271	214	236	269	287	114
F	380	131	378	511	422	441	340	317	289	328	357	359	293	382	305	281	304	351	264	246	309	311	261	114
G	718	882	780	558	482	552	469	519	997	602	420	404	358	400	358	491	281	286	478	612	484	385	151	128
H	778	1178	1018	516	489	786	443	428	811	814	544	776	600	486	817	805	509	508	487	552	507	889	448	176
I	973	704	850	557	664	731	466	691	760	516	548	616	789	659	522	513	528	546	457	504	465	570	475	125
J	1174	1112	1290	1200	661	518	542	698	739	742	604	629	739	742	604	629	739	742	604	629	739	742	604	629
K	1197	1241	1548	536	788	908	387	529	686	517	922	1135	527	625	613	543	583	643	583	659	519	795	488	99
L	1461	1348	1028	881	1397	650	767	742	1088	648	1878	731	703	821	891	640	589	587	743	818	1248	935	484	137
M	1000	1564	1564	321	874	779	889	496	407	884	713	792	388	812	1058	830	718	709	704	570	740	118	716	863
N	1020	1188	585	1216	963	741	988	810	1335	960	892	703	590	792	784	582	393	1083	613	491	841	473	503	101
O	761	181	862	864	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208	1208
P	590	446	1092	855	685	654	1108	977	793	821	1172	810	657	1229	138	646	804	482	698	442	694	558	435	138

Diverset Plate 6

Row\Col	Col																							
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	551	610	804	1663	522	322	1427	905	2116	2986	688	1221	574	1378	873	2848	1629	2041	2636	1479	559	64	1200	3252
B	619	1462	2034	1884	488	1455	2497	1228	1438	1175	1651	2896	2993	2575	1556	1908	2136	3111	2468	2221	1545	2864	2074	1895
C	672	672	1112	1022	986	1147	1817	591	1018	1718	1718	2025	2147	2108	2025	2147	2108	2025	2147	2108	2025	2147	2108	2025
D	1413	750	1107	1518	1507	1988	864	1406	2134	1509	1097	2468	2882	2240	1450	2197	2872	1933	2235	2198	2407	2722	3033	3882
E	1369	1106	1103	1386	1909	1742	2082	2274	2076	2780	2134	2025	2242	1924	1488	1835	1547	1634	1548	2494	2802	2437	2906	4046
F	598	373	276	478	1110	656	1142	1292	1572	1888	954	1068	1158	546	1141	691	1059	1080	2027	1634	1437	1086	2185	1975
G	138	156	156	321	874	779	889	496	407	884	713	792	388	812	1058	830	718	709	704	570	740	118	716	863
H	223	210	140	272	546	488	1012	943	797	1114	954	1156	1025	1136	1690	907	1798	1482	1444	1536	1385	1392	1955	2078
I	265	163	281	221	456	740	429	901	809	752	895	615	968	1097	973	1386	2170	1142	1221	1168	1053	986	2112	1986
J	49	41	39	213	205	742	150	954	436	523	533	202	478	720	210	228	341	828	468	518	545	841	651	228
K	9	39	240	288	703	281	312	387	970	597	587	598	587	1254	914	513	1147	556	730	306	1190	868	1188	1188
L	75	37	315	296	428	438	380	312	387	970	597	587	598	587	1254	914	513	1147	556	730	306	1190	868	1188
M	301	178	215	425	364	374	401	189	757	736	574	439	434	590	507	409	353	386	614	664	637	403	1227	388
N	86	86	186	399	186	186	399	186	186	399	186	186	399	186	186	399	186	186	399	186	186	399	186	186
O	519	614	144	205	799	401	146	693	271	75	239	463	224	184	113	103	65	52	64	272	74	225	237	373

DAPI

Cell Count

Diverset Plate 7

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	245	396	278	396	497	799	501	785	285	792	788	603	1006	801	931	250	350	380	284	471	747	22	28	128
B	622	448	559	343	756	368	541	503	803	519	374	287	279	367	437	551	355	438	408	361	569	299	305	138
C	610	520	610	813	608	512	608	813	608	512	608	813	608	512	608	813	608	512	608	813	608	512	608	813
D	408	644	528	412	341	399	562	667	512	404	541	644	528	412	341	399	562	667	512	404	541	644	528	412
E	1200	439	559	1372	946	988	1309	1637	1342	1557	1200	1778	1949	1408	1627	1623	1452	1686	1472	1333	1485	2308	1278	2151
F	1020	432	1000	1192	1340	988	1309	1637	1342	1557	1020	1409	1600	1278	1778	1409	1600	1278	1778	1409	1600	1278	1778	1409
G	537	87	185	488	822	972	964	704	1305	452	1039	1379	1431	1239	1672	1253	1313	1538	1390	1552	1683	1454	2350	
H	511	158	328	671	606	814	862	1087	1077	1398	718	1087	1288	819	1223	822	1518	1117	769	1411	1005	1308	1652	
I	115	172	711	550	368	912	913	993	993	993	993	993	993	993	993	993	993	993	993	993	993	993	993	
J	20	676	85	428	465	637	1112	722	1721	86	827	1330	931	1323	1048	1438	1453	1390	1295	1655	1499	1372	1733	
K	41	86	419	468	419	468	419	468	419	468	419	468	419	468	419	468	419	468	419	468	419	468	419	
L	229	48	354	207	139	210	228	228	339	544	447	559	600	107	425	528	607	538	554	726	506	1033	638	
M	188	152	213	148	649	188	152	213	148	649	188	152	213	148	649	188	152	213	148	649	188	152	213	
N	32	226	33	217	434	369	914	646	734	602	814	785	438	366	717	404	839	676	829	883	170	1000	842	
O	98	248	197	183	171	197	186	482	325	279	529	181	338	513	299	379	536	82	495	756	381	581	391	
P	158	211	88	1159	393	288	246	334	1000	601	248	1660	1425	413	282	847	86	677	116	288	351	382	599	
Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1161	990	285	1009	417	368	708	609	536	639	772	641	462	310	419	450	577	277	208	439	667	332	557	679
B	580	940	925	382	328	817	421	559	526	421	551	560	462	421	675	467	482	660	443	365	485	800	477	
C	1020	1326	1020	870	760	1326	1020	870	760	1326	1020	870	760	1326	1020	870	760	1326	1020	870	760	1326	1020	870
D	614	727	711	610	354	817	421	871	509	416	428	556	570	999	724	545	485	497	640	354	482	634	355	425
E	408	644	528	412	341	399	562	667	512	404	541	644	528	412	341	399	562	667	512	404	541	644	528	412
F	1200	439	559	1372	946	988	1309	1637	1342	1557	1200	1778	1949	1408	1627	1623	1452	1686	1472	1333	1485	2308	1278	2151
G	726	799	790	432	555	480	400	338	606	835	601	487	461	302	741	100	288	524	611	567	630	377	528	554
H	812	415	812	415	812	415	812	415	812	415	812	415	812	415	812	415	812	415	812	415	812	415	812	415
I	1233	940	792	1002	1005	901	707	530	513	613	791	581	497	518	567	677	481	480	619	683	527	590	638	
J	1111	1338	814	1111	1338	814	1111	1338	814	1111	1338	814	1111	1338	814	1111	1338	814	1111	1338	814	1111	1338	814
K	1461	1350	1333	924	743	1488	766	530	587	525	791	630	414	372	676	600	605	484	556	718	583	482		
L	894	1155	617	1097	1808	905	100	1060	528	605	999	675	961	1293	1041	924	899	483	565	508	528	559	860	
M	851	1376	922	1188	570	870	570	710	644	697	666	765	414	1474	1115	460	629	870	512	511	1332	913	723	
N	1037	992	1064	1134	967	655	979	905	489	646	952	720	1057	932	880	839	717	716	809	667	625	965	556	
O	1248	988	1088	1122	1124	1134	1068	621	588	1068	621	588	1068	621	588	1068	621	588	1068	621	588	1068	621	
P	670	764	727	428	931	1202	734	1182	485	828	568	659	677	681	902	774	130	384	725	759	875	842	681	

TEXAS RED

Diverset Plate 9

Row \ Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	657	900	871	746	1390	1427	2218	1175	247	2288	605	1113	1428	1197	1572	851	912	398	1709	281	774	813	789	841
B	1126	715	1715	1935	1312	2113	1318	1496	1437	1379	1063	552	370	1013	1786	759	1293	518	1104	595	711	734	780	664
C	428	506	2036	78	1330	1342	1311	855	851	1004	1668	1120	1120	1120	1120	841	319	975	248	1302	1841	841	841	841
D	415	825	857	655	823	787	751	1059	1097	749	895	591	388	584	875	561	1393	779	730	540	1079	889	280	144
E	386	537	536	627	1117	1173	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282
F	751	816	1100	957	903	957	1014	913	1446	926	1283	855	980	895	617	943	954	600	587	599	288	430	281	46
G	439	835	730	621	423	1084	485	581	1186	881	841	138	809	1173	811	388	937	751	427	338	580	500	416	127
H	812	182	432	1057	710	459	671	593	707	695	852	652	758	631	851	798	480	397	801	546	487	347	182	182
I	480	656	278	1189	1247	890	819	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282	1282
J	323	1125	1175	283	329	800	1070	1123	1058	674	1059	346	1172	1025	1253	721	982	768	489	653	559	277	324	437
K	826	1286	2139	628	763	2104	111	2758	1316	1079	1158	1044	1361	993	1093	1368	1107	1278	852	246	592	411	253	620
L	949	460	88	336	1395	605	1494	1011	1257	1118	1352	998	974	1037	332	999	980	754	915	594	802	389	230	489
M	681	1884	879	551	364	1246	440	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246	1246
N	3888	818	1090	587	917	730	458	844	856	739	1159	947	609	476	720	599	564	631	1222	283	487	230	225	130
O	682	1689	423	1223	1220	357	353	541	750	767	741	851	759	544	757	477	378	338	444	402	302	344	350	350
P	3892	1020	1129	1458	1276	1459	1853	1292	1290	1361	1537	817	1373	1045	1382	959	936	762	719	536	828	438	878	639

TEXAS

Row \ Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	413	403	427	471	413	331	309	398	409	751	338	490	396	385	408	336	451	457	596	318	644	460	386	440	416
B	390	605	381	347	333	353	561	359	374	388	452	488	588	452	383	479	382	536	430	485	458	512	449	483	483
C	527	564	298	308	327	831	441	479	702	434	462	414	364	328	112	899	518	858	338	433	382	697	598	189	569
D	696	797	554	540	403	447	480	337	385	481	411	543	886	565	515	569	338	486	448	543	472	490	889	677	677
E	467	378	387	395	373	342	287	335	324	402	367	322	355	364	371	389	407	384	433	446	430	447	429	550	550
F	450	515	442	379	426	391	1	368	375	342	386	386	399	391	397	439	437	433	443	452	496	551	522	642	503
G	628	504	477	522	481	437	535	611	375	480	593	885	447	413	442	592	437	459	589	687	551	557	525	833	833
H	38	295	328	1386	476	508	633	560	520	468	541	519	499	468	541	425	511	580	637	521	559	547	686	679	679
I	633	518	809	417	455	590	481	986	477	432	415	428	873	413	402	401	400	413	512	639	539	791	657	718	718
J	485	670	427	503	859	481	481	438	455	485	478	438	458	485	438	429	524	402	475	639	624	534	664	719	728
K	533	565	677	384	554	507	818	609	430	438	588	405	482	424	441	851	523	778	596	601	653	591	653	591	591
L	466	532	853	743	412	487	435	459	400	446	433	523	448	459	661	511	529	518	444	529	536	790	846	679	679
M	634	586	338	1584	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193	1193
N	466	531	505	571	539	587	627	568	545	598	552	601	671	674	610	551	660	672	606	802	697	670	736	618	618
O	508	418	704	457	472	730	679	609	540	443	529	484	308	633	630	445	787	715	758	680	784	735	794	741	741
P	338	464	490	427	381	375	427	388	411	382	428	494	423	426	445	436	506	578	510	596	462	418	497	872	872

Diverset Plate 10

Row \ Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	1965	387	573	644	1254	2768	1589	1880	1114	1040	877	2607	2938	2135	629	1321	789	994	1270	1425	467	1155	1128	1128	1128
B	691	568	1758	752	2833	761	578	844	791	1221	1107	1274	688	1506	871	775	763	750	1261	1270	1299	784	784	784	784
C	117	158	758	543	1382	893	1619	718	1015	1054	2069	1478	918	805	703	607	506	1368	1368	1368	1368	1368	1368	1368	1368
D	795	715	935	652	661	88	965	1609	1601	1314	1372	1042	1131	78	950	631	1459	727	348	839	946	304	449	449	449
E	38	295	328	1386	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157	1157
F	5	303	717	856	1578	727	945	1012	321	744	767	519	1004	1070	237	924	652	674	941	634	723	601	416	416	416
G	16	165	88	1159	520	589	77	633	396	394	302	895	895	895	1036	1226	521	637	789	705	515	38	587	587	587
H	233	535	607	875	946	1013	588	588	680	595	588	514	432	816	317	428	828	845	355	725	596	408	546	546	546
I	56	251	428	922	589	587	1078	680	801	296	1821	466	619	941	647	1208	976	996	890	897	1040	1060	429	429	429
J	524	584	1020	584	1107	772	1341	1219	604	760	604	737	1666	334	969	565	613	608	1153	1397	484	820	1029	397	397
K	41	290	378	1595	1189	1270	868	1318	1365	835	860	1188	811	533	362	468	951	3066	915	840	1085	794	287	287	287
L	3679	1341	790	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260	1260
M	903	822	714	1107	1386	1174	1718	1827	960	1382	1297	1184	1057	1136	1055	996	917	965	996	917	965	917	965	917	965
N	1072	1064	1232	893	1103	1080	1118	1223	914	1057	937	188	1249	685	773	683	882	891	835	902	523	208	208	208	208
O	1180	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045	1045
P	1645	2470	1193	1072	502	1889	1755	1898	1189	317	989	892	881	864	649	1026	507	596	383	460	618	833	592	592	592

Row \ Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	238	423	423	441	311	260	302	290	408	442	412	271	263	285	560	393	513	396	402	364	613	404	381	381	381
B	473	491	318	479	358	422	487	485	451	349	928	389	519	405	427	451	467	470	404	364	352	423	363	29	29
C	705	915	1062	406	331	143	440	547	430	321	289	399	441	409	509	323	470	492	475	475	475	475	475	475	475
D	428	535	442	497	475	852	427	327	358	380	434	381	734	389	461	380	414	732	442	360	404	189	548	24	24
E	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789	789
F	217	479	401	399	304	398	364	376	550	412	412	482	383	366	383	517	369	402	415	374	437	437	437	437	437
G	697	785	767	305	537	449	479	434	408	545	439	618	408	521	361	334	456	405	404	428	444	798	487	487	487
H	642	586	586	456	456	513	523	381	523	381	523	381	523	381	523	381	523	381	523	381	523	381	523	381	523
I	790	766	595	405	498	459	438	561	464	724	335	608	435	448	499	395	445	458	448	458	458	458	458	458	458
J	441	500	476	593	438	488	422	349	501	522	411	340	611	430	579	546	798	428	798	428	798	428	798	428	798
K	651	668	788	389	393	393	472	472	389	393	393	472	472	389	393	393	472	472	389	393	393	472	472	389	393
L	372	366	509	381	455	470	427	341	406	765	409	1069	401	476	517	528	509	516	434	420	591	723	501	501	501
M	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785	785
N	492	495	458	463	444	867	497	405	524	512	472	858	403	625	552	567	571	503	504	475	568	768	776	776	776
O	391	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405	405
P	390	331	472	440	582	298	496	363	398	435	486	473	507	529	572	442	562	538	620	601	564	697	607	607	607

Diverset Plate 13

Row \ Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
A	1016	539	934	396	867	1166	1600	1283	1132	1261	1461	1786	861	1364	1713	1533	1585	1527	2008	2200	1150	1675	1862	2512	2942
B	786	286	904	1176	86	868	951	1031	1360	1180	1160	1564	1746	1442	713	1761	1138	1254	354	1148	1010	1682	3511		
C	902	156	456	507	603	826	837	751	774	1085	978	1039	857	1003	89	1138	950	87	1208	150	1095	961	708	1637	
D	907	651	566	867	832	848	1149	841	865	1002	962	1161	961	752	833	907	633	268	1012	446	1470	868	1010	903	
E	284	716	1189	384	1162	1251	1464	1361	1455	1387	1567	1205	1912	1828	841	1397	1167	1455	2114	1381	1850	1441	1299	907	
F	542	855	1214	1227	1549	1115	1380	1257	867	906	1309	1158	1001	1030	921	1177	1534	1737	879	980	596	1482	1131	1154	
G	825	454	649	765	1012	1290	552	890	1002	893	1118	1104	777	1015	683	888	1124	1140	914	883	1055	1400	1161	2616	
H	1217	779	884	883	1196	923	940	960	1221	924	1007	954	889	951	1332	839	956	804	1385	1150	1032	1293	1103	1222	
I	485	1579	1010	833	1000	1227	1458	1466	902	1309	1075	1044	1478	1520	1527	1088	1356	1152	1219	1380	1352	1295	1206	3288	
J	1140	1148	1188	1090	1055	1097	1151	1146	1304	1427	1305	673	1381	1311	1271	1134	1109	1450	1620	1134	1522	848	1150	2205	
TEXAS RED (Cell Center)	A	888	1158	1129	1380	1194	1177	1323	1740	1474	1306	1312	1312	1136	1088	1008	1532	1329	1540	882	1089	1314	1607	2072	1178
	B	1302	881	1383	1203	1549	1399	1422	942	1128	1848	978	1420	384	1388	1248	1580	1434	1554	1585	1678	1748	1100	2202	7253
	C	1105	1179	1341	131	1396	1080	1192	1504	1088	1115	1466	2000	1328	1302	1054	1763	1707	1010	1282	1042	813	1881	1902	922
	D	1192	1417	1725	1574	1178	905	386	1271	1304	1009	1009	1172	1381	982	1375	1100	1119	1118	1249	1443	1647	1299	1301	6018
	E	212	219	363	1342	378	948	562	722	1076	891	599	1001	1532	778	1042	1213	789	1038	1102	856	835	925	687	2826
	F	436	831	375	288	1136	1221	292	448	1150	548	730	453	1191	507	467	788	422	730	771	615	632	543	1071	695
DAPI (Cell Center)	A	458	522	468	536	513	410	357	400	509	464	397	365	407	398	405	431	388	327	377	345	433	348	348	318
	B	556	585	439	445	491	506	484	474	418	484	448	357	376	430	512	411	438	492	400	655	386	454	366	118
	C	494	796	738	563	564	506	552	491	508	449	542	400	451	451	821	371	358	891	390	570	415	387	409	117
	D	479	552	586	501	533	413	400	599	683	381	414	394	391	479	399	390	516	727	396	523	363	654	487	114
	E	486	400	351	503	359	357	322	355	365	317	339	351	282	339	366	313	339	296	259	297	283	136	335	114
	F	531	512	384	397	347	473	422	386	370	417	388	432	465	464	298	390	357	309	401	407	417	354	438	123
	G	577	660	500	464	368	380	407	416	402	406	430	406	442	443	423	404	364	386	437	475	400	365	438	118
	H	438	538	518	478	493	425	505	429	420	498	436	443	498	419	401	426	467	407	372	389	414	388	409	117
	I	788	378	479	641	435	428	414	420	469	438	436	410	362	369	365	456	368	411	405	389	373	412	386	116
	J	469	449	469	465	365	419	429	426	395	418	474	508	404	396	475	441	425	390	402	427	388	409	407	116
TEXAS RED (Cell Center)	A	357	457	471	420	354	415	408	385	420	416	438	418	427	422	382	394	402	399	445	366	372	355	399	113
	B	435	465	461	464	402	407	437	473	403	388	420	417	865	716	422	394	404	361	380	362	397	410	373	116
	C	519	388	457	788	413	402	415	361	450	484	429	332	405	432	451	385	404	442	459	426	487	385	395	116
	D	438	438	404	460	464	447	464	458	448	447	464	459	479	459	508	508	445	442	394	487	484	487	416	117
	E	754	774	708	452	788	408	602	514	406	419	457	414	373	457	484	371	506	456	487	515	531	473	560	117
	F	442	548	639	784	409	427	709	634	400	644	533	607	471	682	588	513	638	450	498	568	518	436	409	124
DAPI (Cell Center)	A	977	885	1577	1858	1718	1528	1275	177	81	1321	1548	1787	2189	2301	1809	1131	1567	2086	782	2029	2156	2071	1812	1813
	B	759	860	803	1216	1345	1243	1071	138	1121	1214	1071	138	1855	1293	1583	1293	1583	1979	1375	1248	1389	1407	1407	1407
	C	756	630	804	555	754	1000	827	8	910	935	2683	989	2301	797	740	917	896	789	1005	621	910	654	712	438
	D	436	1297	27	1089	479	1304	1121	1478	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740	1740
	E	78	484	333	1439	1151	953	949	838	952	812	838	1206	1395	1039	1279	1295	1153	1254	1441	1647	1478	1221	1414	750
	F	4697	795	681	826	826	267	328	328	328	328	328	328	328	328	328	328	328	328	328	328	328	328	328	328
	G	1271	587	1117	954	1150	1264	1180	974	1506	805	860	1177	1356	989	1013	634	1101	1303	788	1181	1447	1245	1436	1529
	H	1229	1422	283	1179	1319	1509	1386	283	1376	1593	1370	1244	1580	584	935	1277	865	1457	1521	1786	1240	1489	1977	2529
	I	1487	706	1010	1005	1339	1385	1030	1832	1728	1539	1538	1477	1599	1071	500	1457	188	1380	1483	1464	1891	1763	1756	2158
	J	3984	1110	1471	1576	1661	1545	1560	1562	1564	1324	1661	1723	1117	1271	1579	1387	1387	1387	1387	1387	1387	1387	1387	1387
TEXAS RED (Cell Center)	A	536	1035	1495	808	1313	684	1748	1484	1176	1414	1502	1507	730	1476	108	1240	765	1397	2111	2084	2281	1963	2387	3428
	B	2076	618	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131	1290	1131
	C	270	518	767	563	810	783	796	539	697	575	1212	998	805	1296	874	1418	1177	1028	1407	1457	1036	1476	1512	2041
	D	344	838	338	656	420	261	548	537	1330	1108	338	805	818	522	12	400	1296	886	4	1123	2072	1804	3352	

Diverset Plate 17

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	746	757	619	1478	469	378	569	1830	891	1066	1641	1460	1892	1822	1495	1450	1970	2658	1354	1521	1394	1441	1617	3406
B	318	334	338	836	547	1158	1136	815	806	1095	1073	837	1852	324	588	1389	939	878	1794	845	811	785	1274	278
C	138	382	35	140	42	923	1446	290	296	330	376	549	1189	821	396	799	437	388	1146	458	1211	1615	901	936
D	600	757	723	644	1106	438	1079	1006	738	814	1059	984	1370	1421	859	1213	688	1156	1233	1377	1383	1260	1020	723
E	25	707	278	1592	1293	2095	1556	837	1425	1881	2340	1222	1591	696	2070	2266	1605	1022	2217	1620	2215	1761	1996	356
F	1042	728	1228	882	1251	1733	1947	1643	1035	2873	1733	2878	2436	2021	1234	1884	2648	2339	2383	1502	1992	2302	1506	568
G	470	222	81	658	564	832	837	658	534	527	484	1312	1840	388	1452	967	669	863	761	1169	1360	3996	2384	1578
H	1246	438	479	356	317	622	382	796	787	647	1136	562	747	1006	982	1151	1148	1382	379	1266	1250	1348	1287	1247
I	38	38	356	1153	967	1217	1545	1235	1597	2447	390	2599	2172	3958	1590	2054	2538	1552	1733	2485	1478	2300	2888	1680
J	1308	1186	1284	1782	1409	1818	1282	1663	1864	1774	356	2229	2754	1891	2349	1866	2371	2218	2879	3254	3388	2858	2120	1307
K	1381	728	275	712	386	1045	640	1042	599	1246	778	1981	2088	1664	1556	1114	328	2201	2488	1739	1742	1073	1294	1284
L	986	508	628	1050	881	1499	1987	1238	1672	2000	1688	2236	2511	2136	875	973	1421	2385	1958	2124	2209	1766	2145	1047
M	728	700	328	984	328	1038	1156	1539	1138	1896	1804	2454	2848	1993	1633	1990	2209	772	2307	2058	2569	1948	2834	3338
N	138	1672	1672	2078	1452	2984	2126	1698	1389	1611	2377	1754	2126	1859	1735	1812	1865	1793	1643	2154	1685	2662	2469	2725
O	1105	915	381	967	323	1383	1209	1701	1727	1275	2281	1448	1761	2754	1349	1904	2431	562	1489	2185	1641	986	2142	88
P	1456	1209	597	2096	1552	658	1787	1197	1303	2398	1403	632	1971	2447	1534	3628	1523	1863	3854	1382	1886	2195	2591	2947
Average Column	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
A	320	466	333	272	458	494	411	323	451	352	393	360	365	320	331	418	339	271	276	406	351	416	470	217
B	798	443	702	463	539	363	370	419	497	348	344	402	310	511	441	366	431	424	298	412	651	445	399	115
C	717	518	899	746	748	425	321	628	495	567	668	487	381	473	552	431	475	628	345	483	341	339	353	118
D	452	483	446	439	950	564	352	358	444	413	384	367	324	278	382	345	429	438	817	305	301	329	369	139
E	427	372	438	283	316	260	274	331	312	270	241	245	288	383	251	258	272	288	238	251	265	262	289	59
F	427	365	363	345	367	321	317	307	328	350	311	243	298	309	315	290	282	281	248	314	304	272	304	159
G	486	693	928	475	588	546	501	444	426	516	509	327	302	322	329	388	453	403	432	356	350	302	279	114
H	332	496	466	637	899	496	511	464	433	531	392	448	489	352	390	363	353	368	500	368	330	327	368	116
I	288	370	636	325	402	403	302	348	350	311	412	265	276	280	302	275	276	317	313	278	330	320	245	136
J	506	364	408	315	355	329	388	354	296	322	700	252	265	303	297	291	270	281	229	246	237	279	314	116
K	409	432	666	421	568	421	458	361	397	356	437	306	280	348	351	341	492	513	298	277	304	304	298	157
L	462	574	554	424	375	325	311	310	324	289	339	293	293	333	346	411	326	256	280	299	280	311	314	116
M	527	477	558	390	386	450	400	331	399	334	298	297	300	335	336	308	348	489	320	308	288	331	285	116
N	786	385	361	332	434	312	338	338	405	381	330	346	294	305	334	322	337	323	336	324	370	361	305	136
O	369	454	552	473	262	375	366	304	290	339	266	334	328	300	338	289	315	424	334	290	331	445	283	117
P	381	346	438	301	325	568	340	413	355	299	314	474	393	367	280	227	348	316	246	390	338	264	255	137

Diverset Plate 18

Row\Col	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	546	338	150	191	713	1209	481	637	1175	1159	1631	855	1730	1150	2247	1063	1257	1641	678	1126	238	1281	1985	2190
B	289	36	335	363	148	284	600	733	550	388	461	738	762	482	799	611	629	388	840	550	1074	388	608	373
C	693	258	162	245	605	606	781	234	388	802	1184	365	814	641	1022	541	882	1261	808	1127	1458	1263	915	1736
D	415	488	375	380	505	268	446	268	595	553	58	755	1021	737	358	962	821	849	1201	38	683	1205	613	225
E	308	53	690	1096	2286	717	815	1313	2078	1544	1170	2289	1762	1828	2005	1639	2279	1648	1642	2158	2286	1485	1281	22
F	398	638	618	559	839	1562	727	1253	844	936	1925	1522	1336	2324	1435	1254	1808	348	2462	1053	2147	2208	1150	189
G	954	347	425	471	786	624	288	348	890	384	468	528	1161	388	700	1704	947	321	1774	2265	1456	755	1684	58
H	672	337	277	340	563	36	387	796	388	645	847	388	1078	732	548	1006	878	999	992	1346	600	1364	1099	1645
I	1261	434	806	1164	658	1270	1220	1545	1642	1275	1122	1887	1729	1647	1140	1892	1762	1072	1674	2769	2639	785	2189	1229
J	1097	335	1149	918	1104	1342	1266	1577	657	1514	1747	1180	1569	1892	1616	1932	2487	1393	1852	1462	1522	1852	870	1670
K	704	219	587	725	798	899	1158	1840	1867	1626	2159	2045	1552	1380	1134	898	1879	1538	1624	1645	1093	2688	1731	1607
L	1853	395	719	489	1447	1171	1164	1821	1476	1440	2255	1613	1561	1268	1814	1599	1794	898	1620	1487	1757	1348	1500	638
M	1048	352	785	1394	1350	1090	1973	1636	1561	1459	2057	1631	2538	2057	2051	1952	2105	2329	2215	2367	222	1476	1551	298
N	765	324	355	1120	1156	1701	2117	78	755	1823	2888	1261	2660	1954	1916	379	1591	388	2150	827	874	474	1415	298
O	1727	1209	1214	1203	2406	2288	1850	2752	1626	2708	1936	2500	652	2227	2409	1590	2281	2425	1921	2605	2558	2684	1743	2211
P	1698	1220	2177	828	2352	1294	1338	2145	2388	1893	1722	1506	1841	1116	2861	2340	2640	3139	2571	3881	3124	2691	2495	1508
Average Column	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
A	540	595	796	613	563	397	557	405	340	327	354	422	395	390	311	395	345	285	491	394	678	314	319	318
B	524	325	750	563	641	758	434	657	526	656	488	679	502	544	500	472	503	427	444	563	410	443	437	518
C	494	722	748	619	465	513	439	611	738	295	367	868	413	403	351	489	399	325	382	375	373	337	337	174
D	611	390	596	549	481	688	520	647	567	471	138	950	372	405	378	489	440	373	315	798	420	388	386	139
E	520	387	398	351	289	390	387	303	260	285	312	265	295	270	272	289	291	290	341	277	298	319	319	54
F	698	413	409	455	627	343	466	397	427	384	345	250	353	259	276	303	295	336	288	351	399	362	389	157
G	501	639	572	426	398	451	619	626	409	589	522	549	349	437	478	327	349	394	335	399	336	445	323	117
H	483	532	665	604	523	828	438	446	368	446	400	688	435	311	311	334	384	400	400	350	458	351	374	118
I	345	367	427	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367	367
J	356	616	434	356	463	380	436	347	507	372	372	328	399	538	334	371	327	304	346	345	399	380	311	323
K	345	669	414	335	431	460	439	313	363	341	288	311	387	380	378	546	323	367	316	326	397	282	352	371
L	355	608	608	355	438	468	388	355	412	318	318	412	387	386	412	387	386	412	387	386	412	387	386	412
M	446	628	502	409	428	352	316	343	356	326	343	356	326	328	346	334	359	316	316	318	322	668	363	337
N	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324	324
O	328	385	416	418	387	324	327	380	338	367	281	333	343	534	350	305	369	340	326	319	286	276	289	377
P	316	713	533	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333	333
Average Column	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11

Supplementary Figure 2

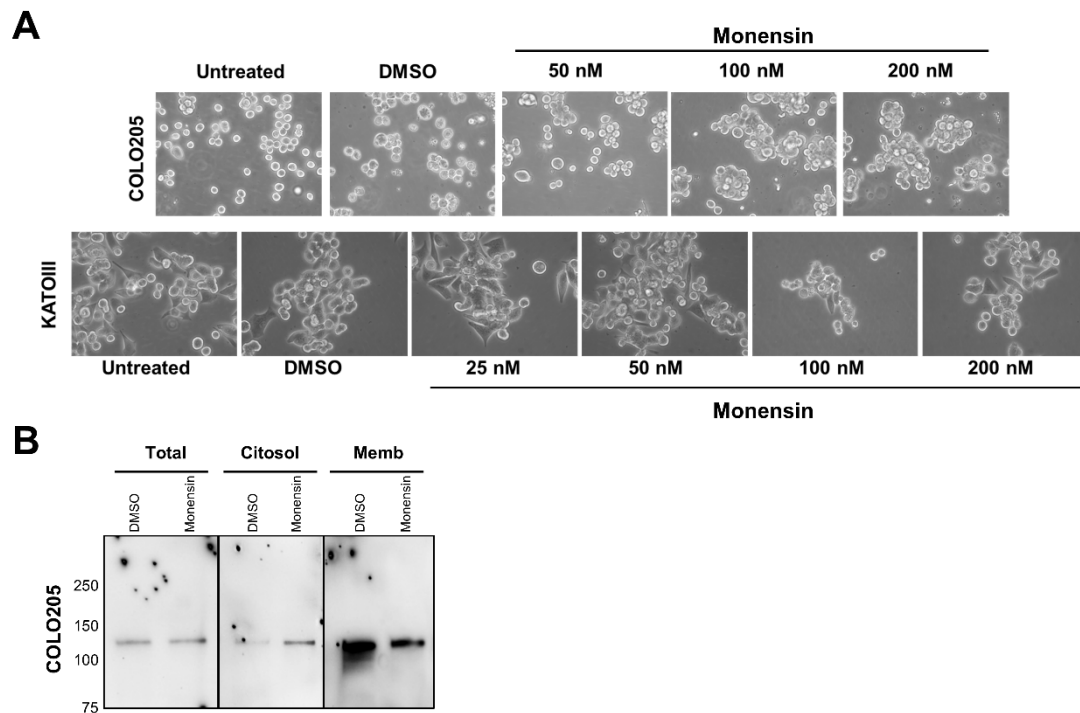
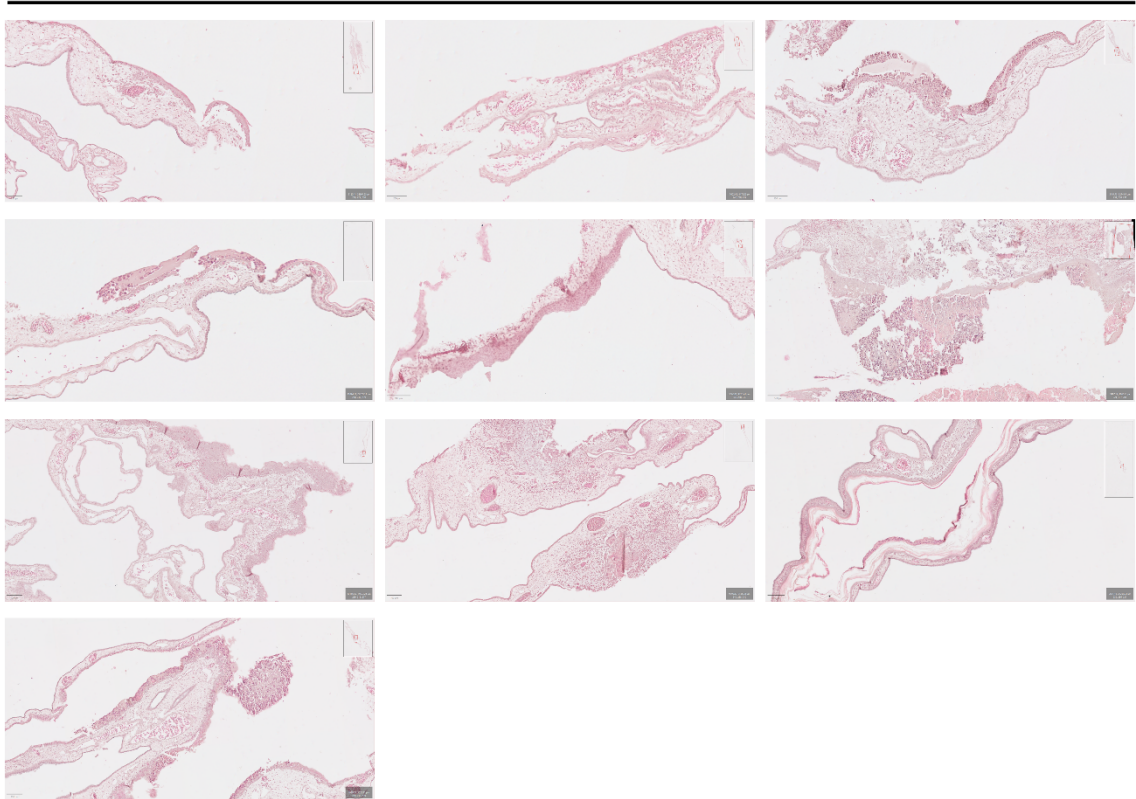


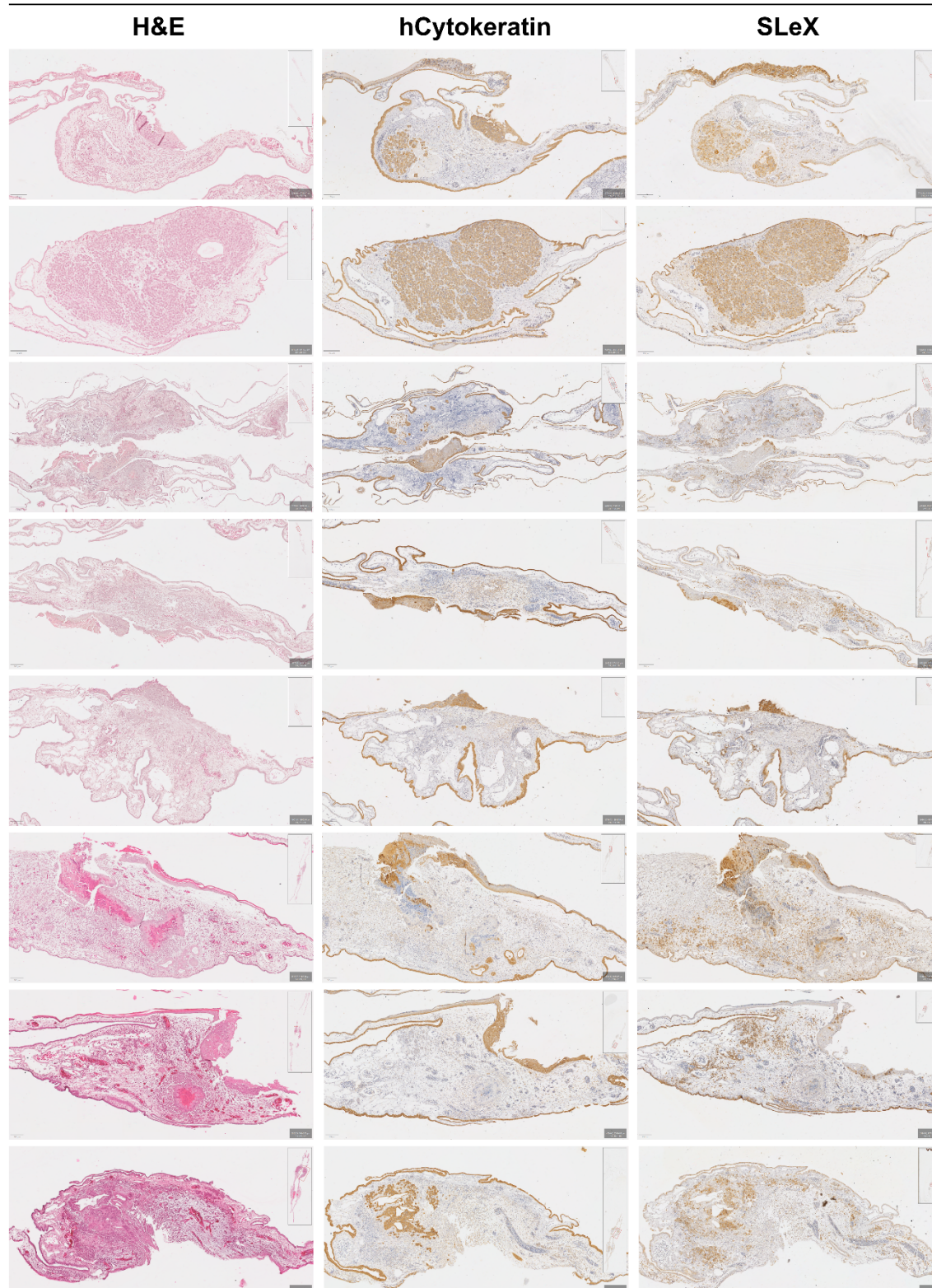
Figure S2: Effect of monensin treatment on CRC and GC cell morphology and in E-Cadherin expression in CRC cells. A) Brightfield microscopy images upon 72h of monensin treatment showing morphological alteration in both COLO205 and KATOIII treated cells compared to the untreated or DMSO treated cells. It is observed a larger vacuolar cell phenotype and an increase in cells' aggregation upon monensin treatment (Magnification 200x). **B)** Western blot analysis of E-cadherin expression at different cellular compartments (total, cytosolic and membrane) in COLO205 cells. Monesin treatment led to a reduction of E-cadherin expression at the membrane fraction and an increase at the cytosolic fraction.

Supplementary Figure 3
A

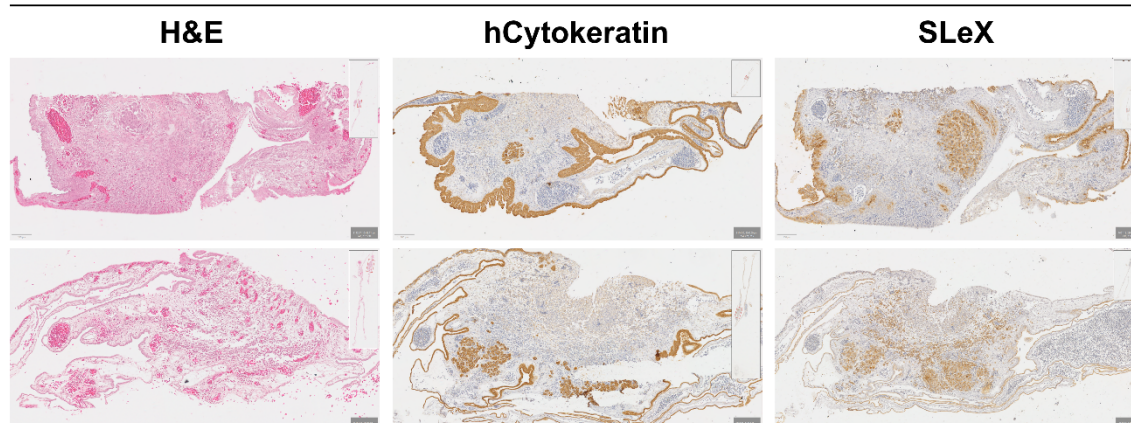
KATOIII
DMSO

H&E

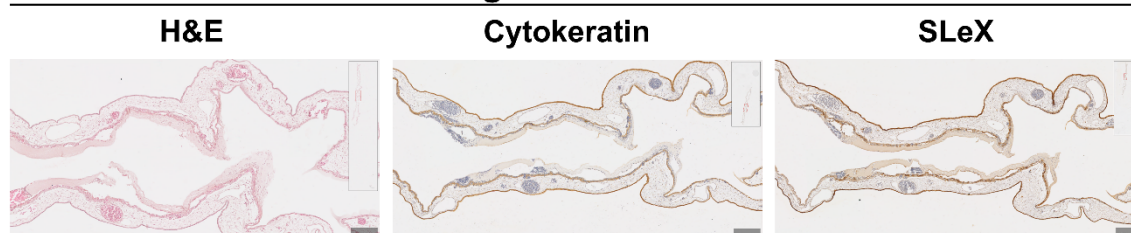


B**DMSO****COLO205**

DMSO

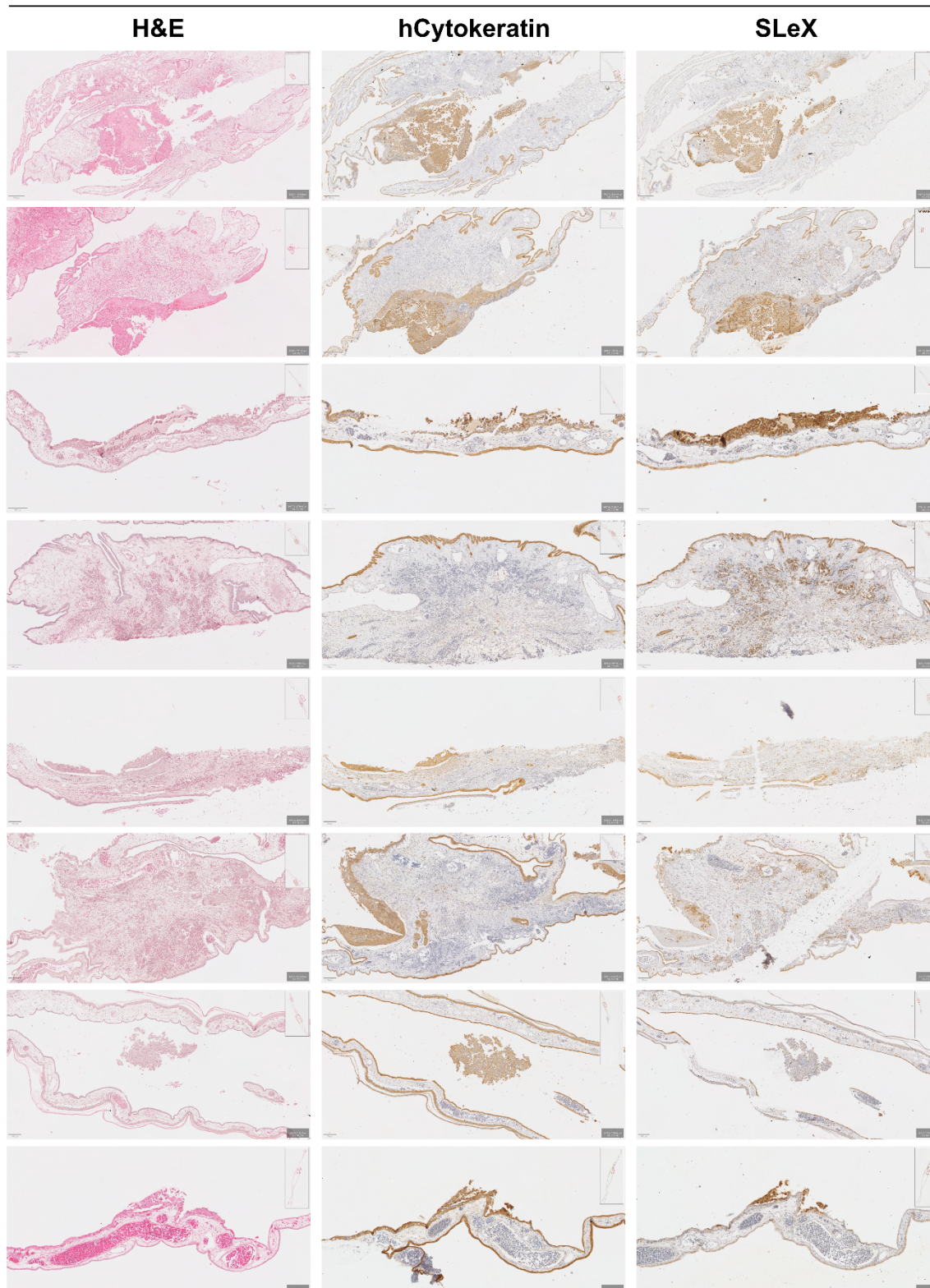


Negative control



Mon 200 nM

COLO205



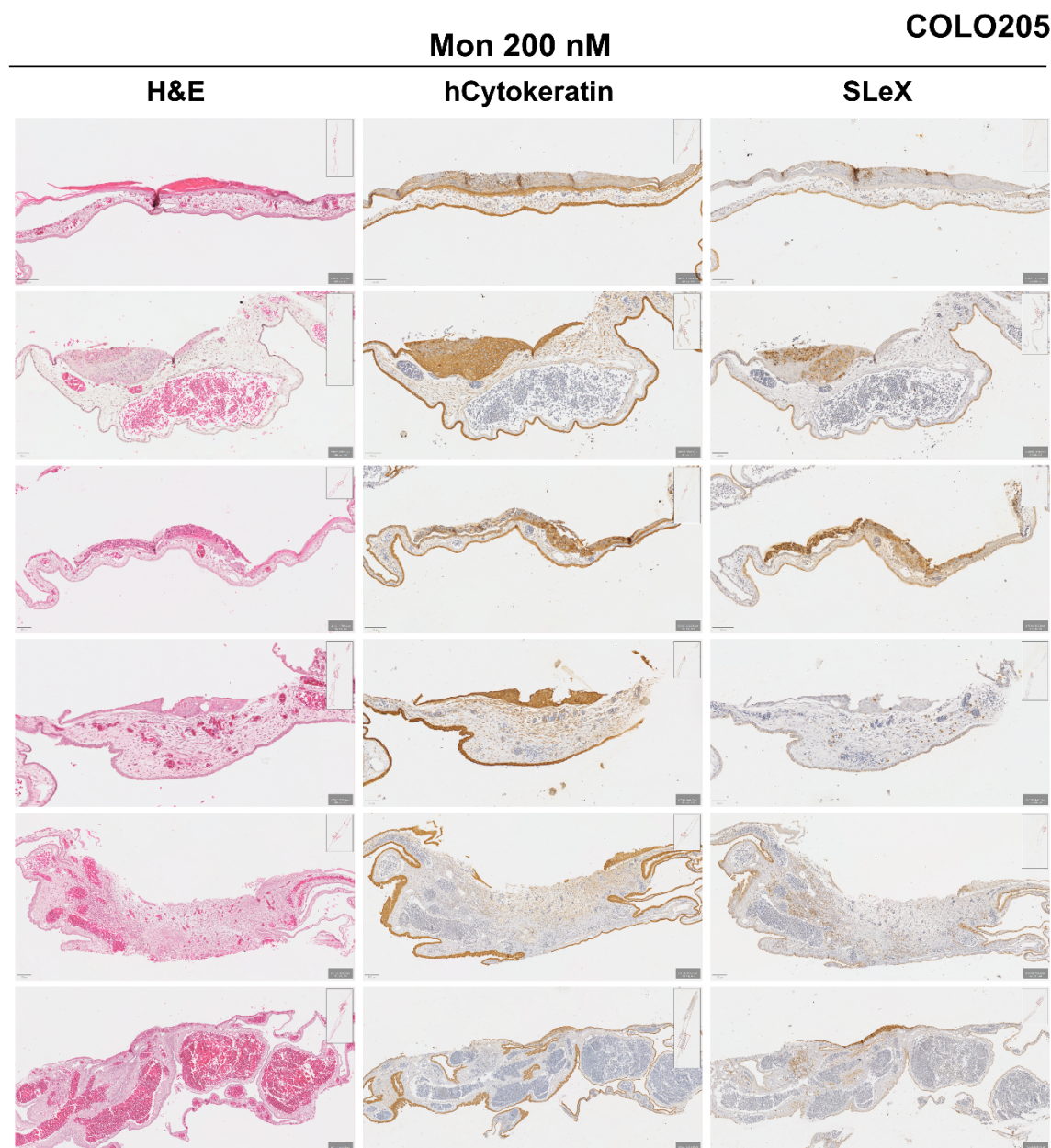
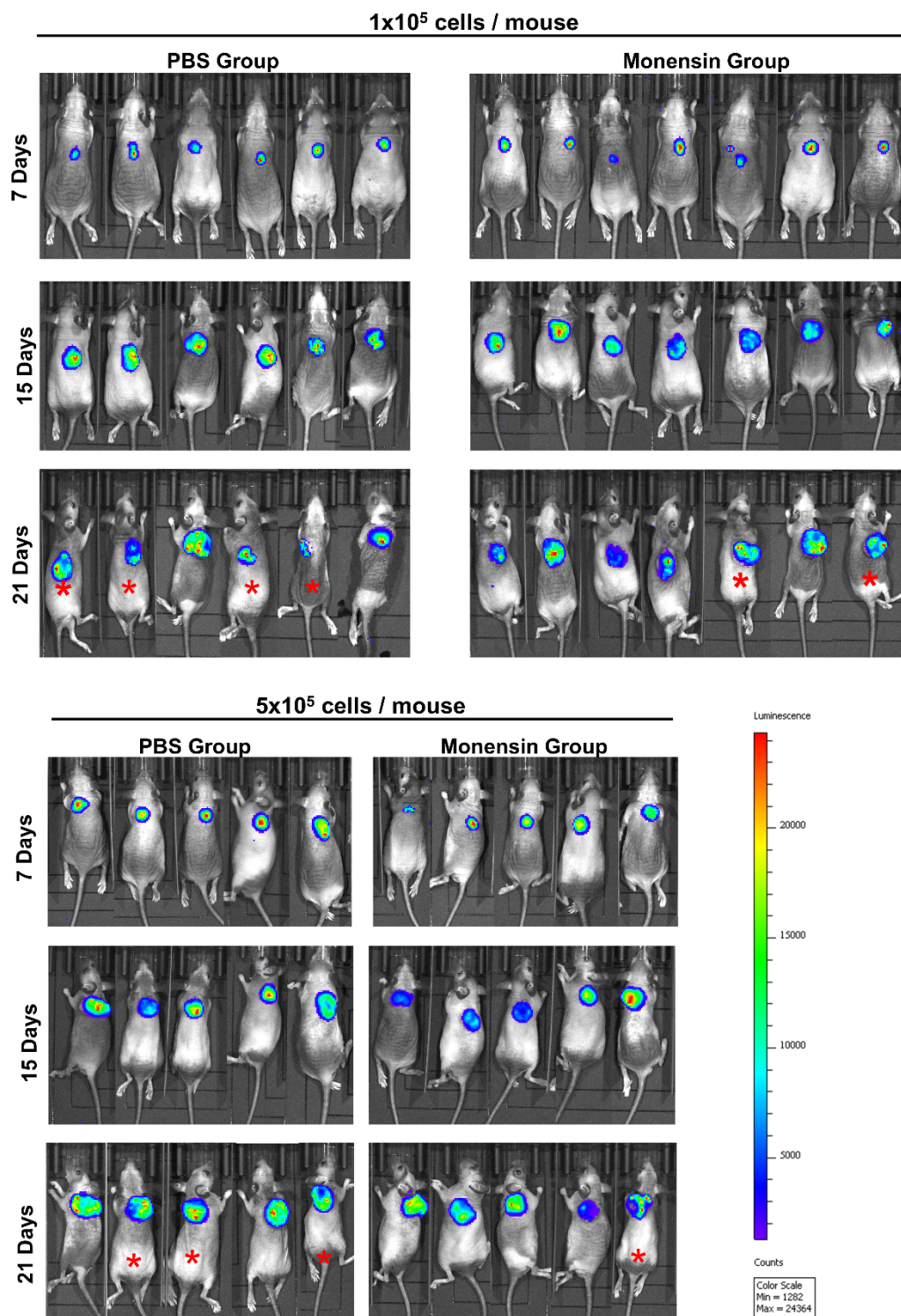


Figure S3: Histological analysis of chick chorioallantoic membrane (CAM) xenografted with KATOIII and COLO205 cell lines. A) Hematoxylin & Eosin (H&E) staining of KATOIII xenografted CAMs on day 6 showing no tumor formation. **B)** Histological characterization (H&E, human cytokeratins and SLeX) of COLO205 xenografted CAMs showing tumor formation at day 6 in DMSO-treated, monensin-treated and vitrogel control CAMs. Results show differences in tumor size and cell invasion throughout the chicken membrane in monesin-treated CAMs. Scale bars correspond to 50-200 μ m.

Supplementary Figure 4

A



* The animal's tumor ulcerated shortly before the end of the experiment (1-2 days before), which led us, in some cases, to use the last measurement before the ulcer occurs.

B

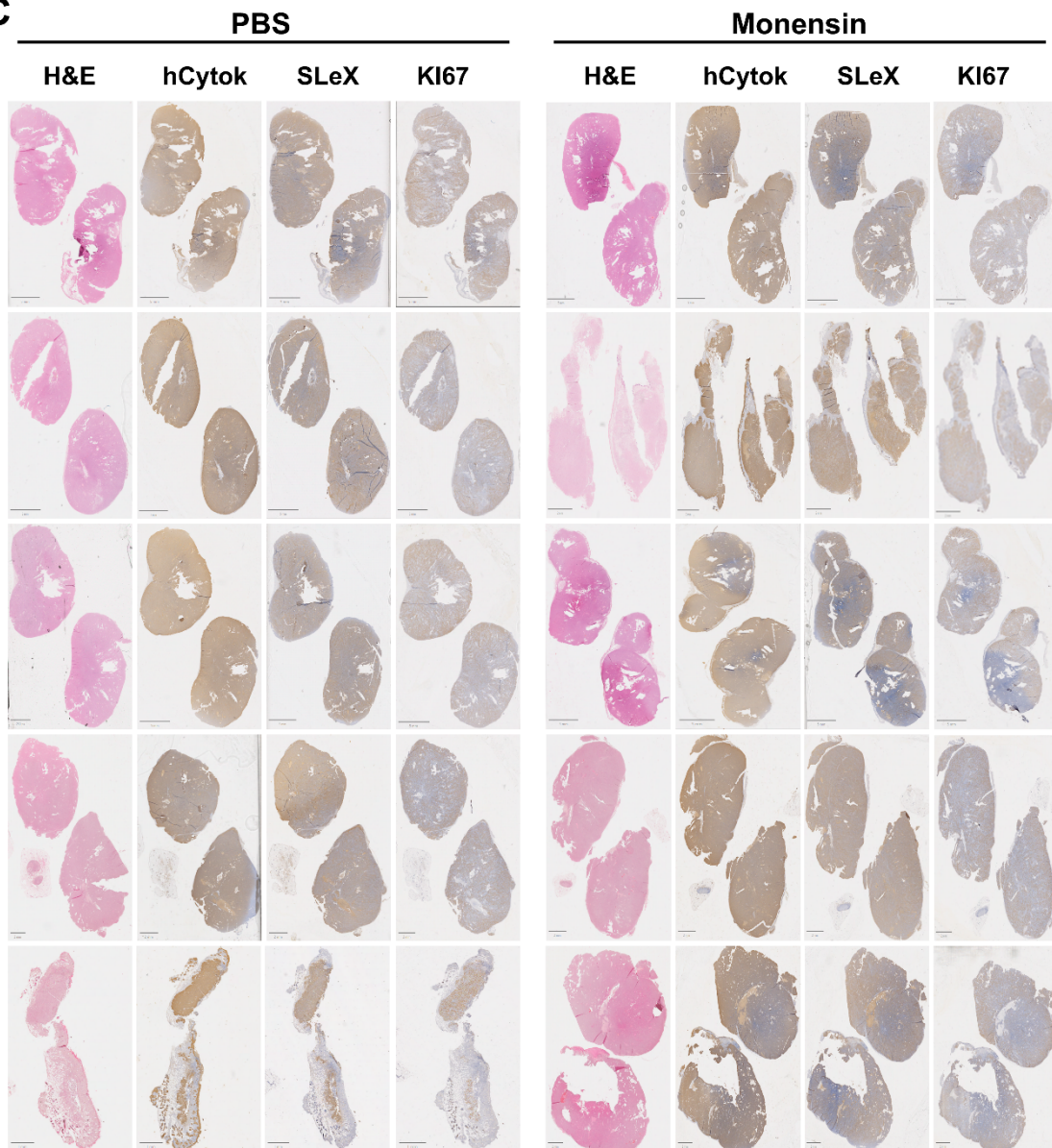
C**5x10⁶ cells/mouse**



Figure S4: *In vivo* biological effect of monensin in Luciferase-positive COLO205 xenograft mouse model using 1 million and 5 million cell inoculums. A) In vivo tumor growth by measuring luciferase activity throughout time. **B)** Tumor images of Hematoxylin & Eosin (H&E) staining and immunodetection of human cytokeratin, SLeX and KI67 in mice subcutaneously injected with 1 million cells. **C)** Tumor images of Hematoxylin & Eosin (H&E) staining and immunodetection of human cytokeratin, SLeX and KI67 in mice subcutaneously injected with 5 million cells. **D)** H&E and human cytokeratin staining of livers from mice injected with 1 or 5 million cells showing no metastatic lesions.

Supplementary Figure 5

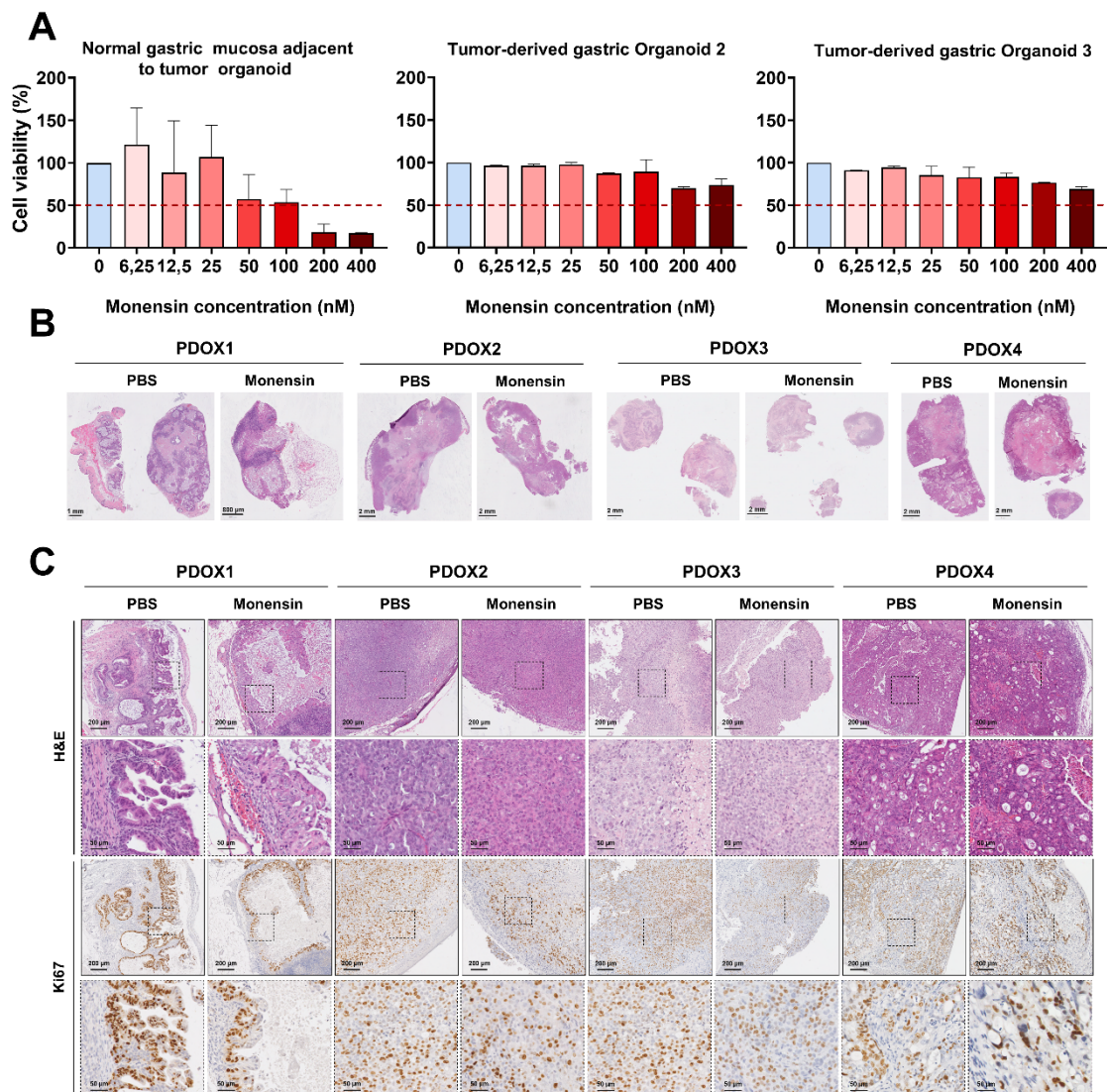


Figure S5: Monensin biological effect in a pre-clinical organoid model. A) Viability assay of healthy (left) and two tumor-patient (right) derived gastric organoids after monensin treatment. **B)** Tumor's images (H&E staining) of PDOX DMSO and monensin-treated **C)** Detailed histological characterization, H&E and Ki67 immunodetection, of PDOX tumors.

Chapter 4

4F-GalNAc is a general inhibitor of cancer-associated O-glycans in gastrointestinal tumors

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4F-GalNAc is a general inhibitor of cancer-associated O-glycans in gastrointestinal tumors

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Abstract:

The aberrant expression of sialylated O-glycans, such as STn and O-linked SLeX antigens, is a common feature in gastrointestinal cancers (GIC). Monosaccharide analogs have been showing substantial impact as novel inhibitors of glycosylation. Thus, this study aims to evaluate the capacity of different fluorinated *N*-acetylgalactosamine (GalNAc) in impairing the biosynthesis of aberrant O-glycans in GIC cells as promising drugs. We tested three distinct fluorinated GalNAc monosaccharides, with a fluorine atom at the positions -3, -4, and -6 (3F-, 4F- and 6F-GalNAc), in natural-expressing SLeX and Tn/STn glycoengineered cell models (Δ C1GalT1) and evaluated their impact in the glycan biosynthesis and cancer biological characteristics. Overall, our results demonstrated that 4F-GalNAc led to a reduction in the immunodetection of both O-linked SLeX and Tn/STn antigens, along with alterations in genes involved in *N*-glycan biosynthesis and sugar donor metabolic activity. This effect impacted the motility and invasive capacity of cancer cells, decreasing E-selectin binding and altering the activation of cell surface receptors tyrosine kinase (RTKs). In addition, *in vivo* results showed the capacity of 4F-GalNAc to reduce tumor growth in a xenograft mouse model. Our results indicate that 4F-GalNAc can disrupt cancer-associated O-glycans biosynthesis, affecting associated malignant biological features, constituting a potential novel approach for developing a new cancer therapy.

Keywords:

Cancer Therapy; Glycosylation inhibitors, Fluorinated monosaccharides analogs; 4F-GalNAc

Introduction

Gastrointestinal cancers (GIC), including gastric cancer (GC) and colorectal cancer (CRC), rank among the top five deadliest cancers worldwide [1]. The currently used therapies present limitations, due to their ineffectiveness in several groups of patients, therapy resistance, and adverse side effects. This frequently leads to tumor recurrence, metastasis, and ultimately patient death [2,3]. Therefore, there is an urgent need to explore novel molecular targets to develop innovative anti-cancer agents with better therapeutic efficacy.

One of the common features of cancer is the aberrant expression of sialylated O-glycans [4]. The truncated O-glycans Tn (GalNAc-O-Thr/Ser) and Sialyl Tn (STn, NeuAc α 2-6GalNAc-O-Thr/Ser) and O-linked sialylated Lewis structures such as Sialyl Lewis X (SLeX, Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc-R) are often associated with more aggressive cancer phenotypes such as migration, invasion, and metastasis formation [5]. Consequently, the presence of these aberrant glycans is linked to a poor prognosis for patients [4-9]. Therefore, impairing the biosynthesis of these aberrant glycans could present a promising therapeutic approach to halt cancer progression and aggressiveness [2,5].

A recent and promising approach involves the use of metabolic inhibitors, such as chemically modified sugar analogs. An example is the fluorinated monosaccharide analogs (F-MAs), which involve the chemical replacement of one or more hydroxyl groups (-OH) attached to the sugar carbons of natural monosaccharides with fluorine atoms [10]. F-MAs, such as 2F-Fuc and 3F_{ax}-Neu5Ac, have shown significant potential in inhibiting both fucosylation and sialylation, displaying promising anti-cancer effects both *in vitro* and *in vivo* [11-14]. Notably, the orally bioavailable 2F-Fuc reached a Phase I clinical trial for patients with advanced solid tumors (NCT 02952989), with great potential in suppressing tumor growth [15]. Fluorinated HexNAc (GlcNAc and GalNAc) are comparatively less explored, however, 4F-GlcNAc has been the focus of several studies. This F-MA impacts general glycosylation, reducing the expression of selectin ligands (Sialyl Lewis A (SLeA) and SLeX), impairing cell adhesion, reducing tumor growth, and hindering cancer cells' immune evasion [16-20]. On the contrary, fluorinated GalNAc analogs are less studied, with 4F- and 6F-GalNAc demonstrating the capacity to impair glycosylation, mainly O-glycans and glycosaminoglycans (GAGs) biosynthesis [20-22]. The mechanisms of action of these F-MAs have not been fully elucidated with authors claiming their incorporation into glycans structures of multiple glycoproteins compromising further modifications [21], while others suggest an inhibition of endogenous sugar donor metabolic pathways [20,23]. These shreds of evidence warrant further research on fluorinated HexNAcs as they hold great potential as cancer-associated glycosylation inhibitors.

In this study, we evaluated the capacity of three distinct fluorinated GalNAc (3F-, 4F- and 6F-GalNAc) to impair the biosynthesis of cancer-associated O-glycans. We showed that only 4F-GalNAc can inhibit both O-linked SLeX and Tn/STn antigens in GIC cell lines and their associated malignant properties *in vitro* and *in vivo*. RNA sequencing (RNA-Seq) results highlighted alterations in sugar metabolic pathways and *N*-glycosylation biosynthesis.

Materials and methods

Cell lines and cell culture

COLO205, KATOIII and LS174T were obtained from the American Type Culture Collection (ATCC) and MKN45 was obtained from the Japanese Cancer Research Resources Bank. Cells were grown in monolayer culture in 6-well plates (Fisher Scientific) and maintained at 37 °C in an atmosphere of 5% CO₂, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX HEPES medium, Dulbecco's Modified Eagle Medium (DMEM) or Iscove Modified Dulbecco Media (IMDM) (all from Biowest), supplemented with 10% fetal bovine serum (FBS) (Biowest).

CRISPR/Cas9 knockout

C1GalT1 Knock-Out (KO) was performed using CRISPR/Cas9 technology as described previously [24]. Briefly, cells were co-transfected with a Cas9 endonuclease vector containing GFP and a plasmid with a validated guide RNA for the *C1GalT1* gene "GTAAAGCAGGGCTACATGAG". KO clones were obtained by fluorescence-activated single-cell sorting (FACS) for the enrichment of nuclease-expressing cells, and gene KO was validated by indel detection using amplicon analysis by restriction fragment length polymorphism (RFLP), as previously described [25]. Three clones containing distinct INDEL were selected, and gene KO was confirmed by Sanger sequencing. Indels were analyzed using the Tracking of Indels by Decomposition (TIDE) assay [26].

F-MAs synthesis

3F- and 4F-GalNAc [27], 6F-GalNAc [20], and 3F_{ax}-Neu5Ac [11] were synthesized as described previously. All compounds were dissolved in DMSO and stocked at -20 °C at a final concentration of 60 mM.

Dose-response treatment with F-MAs

For dose-response assays, cells were seeded in 12-well plates 24 h before treatment with

increasing 3F-, 4F-, and 6F-GalNAc or 3F_{ax}-Neu5Ac concentrations (18.75, 37.5, 75, 150 and 300 μ M) for 24 h, 48 and 72 h.

Flow Cytometry analysis

Cells were detached using accutase (ThermoFisher), spun down at 300 g for 5 min at 4 °C. Afterwards, they were stained with SLeX antibody (CSLEX, dilution 1:100, BDBioscience), SLeA antibody (CA19.9, dilution 1:200, Abcam), STn hybridoma (3F1, undiluted) and Tn hybridoma (5F4, 1:2) for 30 min on ice. Cells were washed with FACs Buffer (BSA 1%) and incubated with secondary antibody Alexa 647 anti-mouse IgM (dilution 1:400, Jackson ImmunoResearch) for SLeX and Tn mAbs, and with Alexa 488 anti-mouse IgG (dilution 1:500, Jackson ImmunoResearch) for SLeA and STn mAbs, for 30 min on ice in the dark. Cells were stained with DAPI (10 μ g/ml, Sigma-Aldrich) for 10 min at room temperature in the dark. Samples were analyzed on a FACS Fortessa Flow cytometer (BDBioscience) and FlowJo software of analysis.

PNGase F digestion

Whole-cell lysates were obtained from confluent 6-well cell cultures using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined by the bicinchoninic acid protein assay (BCA) (Pierce). Denaturation buffer was added to the lysates and incubated at 100 °C for 10 min. Then, the lysates were digested ON with 1 unit of PNGase F according to the manufacturer's instructions (New England Biolabs) at 37 °C. PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high-mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins.

SDS-PAGE and Western Blot

After protein concentration quantification through BCA assay (Pierce), 5–10 μ g of protein extracts were loaded onto 4–15% Mini-PROTEAN®TGXTM precast polyacrylamide gels (Bio-Rad) for electrophoretic separation (SDS-PAGE) (Bio-Rad). Gels were then transferred onto nitrocellulose membranes (Amersham) and blocked for 1 h with 5% bovine serum albumin (BSA) (Sigma-Aldrich) diluted in PBS containing 0.05% Tween 20 (Sigma-Aldrich) (PBS-T). For western blotting, the membranes were incubated ON at 4 °C with SLeX antibody (CSLEX, dilution 1:500, BDBioscience), STn hybridoma (3F1, Undiluted), and Tn hybridoma (5F4, 1:2), followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgM (dilution 1:15000, Jackson ImmunoResearch) for SLeX and Tn, and a horseradish peroxidase-conjugated goat anti-mouse IgG (1:25000, Jackson

ImmunoResearch) for STn during 1h. Protein bands were visualized using the ECL WB detection reagent and ChemiDoc Imaging System (Bio-Rad).

RNAseq

Cells were seeded in 6-well plates and allowed to attach for 24 h. Then, cells were treated with 4F-GalNAc (75 μ M for COLO205 WT and Δ C1GalT1 cells, 150 μ M for KATOIII) or with DMSO (vehicle) for 48h. Cells were harvested and RNA extracted using Pure LinkTM RNA Mini Kit (Invitrogen) according to manufacturer protocol. The quality of the RNA was first evaluated by NanoDropTM One Spectrophotometer (ThermoFisher Scientific) and the samples were stored at -20 °C until use. Three biological replicates from each condition were guaranteed. The RNA was sent to NovogeneCo., Ltd. for RNA quality control followed by cDNA library construction, sequencing, and bioinformatic analysis. The quality of the paired-end sequencing reads was checked using FastQC and the low-quality reads and adapters were removed using Trimmomatic. The pre-processed reads were mapped to the reference using HISAT2. The resulting read alignments were then used to generate the gene counts using the feature Counts software and normalised to FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced). Differential gene expression (DEG) analysis of the gene counts data was performed using DESeq2 R by means of a differential gene screening threshold of $|\log_2(\text{FoldChange})| \geq 1$ & $\text{padj} \leq 0.05$. Through the enrichment analysis of the differential expressed genes, the enrichment analysis on gene sets (GO) was obtained using the clusterProfiler software.

MTT cell viability analysis

Cell viability was analyzed *in vitro* by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using a commercially available kit (MTT Cell Proliferation Kit I, Roche). Cells were seeded into 96-well plates at the density of 5×10^3 cells/well, treated with 4F-GalNAc (75 μ M for COLO205 WT and Δ C1GalT1 cells, and 150 μ M for KATOIII), or DMSO as control, and MTT Kit solutions added into each well according to cell datasheet kit instructions (Roche) at each time point 24, 48 and 72 h. The absorbance value was read at 600 nm using a microplate reader (Biotek). Three independent assays were performed in triplicates and results are presented as means $\pm$ SEM for each sample. The viability levels obtained were normalized and compared with the DMSO treatment control.

Wound Healing Assay

Cell mobility was assessed *in vitro* by a wound-healing assay. Approximately 1.4×10^6 cells were seeded into IBIDI 3-well inserts in 24-well plates until reaching confluency.

The inserts were removed, and cells were washed twice with PBS. Cells were treated with 4F-GalNAc (75 μ M for COLO205 WT and Δ C1GalT1 cells, and 150 μ M for KATOIII), and DMSO as control, and were imaged at 0, 24, 48 and 72 h at a magnification of 100 $\times$. Cell migration was calculated as the mean percentage of the cell migrated distance compared with the initial wound distance using ImageJ software (FIJI). The experiment was performed in triplicate in three biological replicates and motility capacity compared within the different conditions.

Invasion Assay

Invasion assays were performed in a BD Biocoat Matrigel invasion chamber (Corning) with an 8-mm diameter pore size membrane with a thin layer of Matrigel, in a 6-well plate. Inserts were rehydrated for at least 1 h with culture medium. After detachment of confluent cells with trypsin/EDTA, 9×10^5 cells were seeded in the upper surface of Transwell plates and cultured in i) culture medium containing 10% FBS and DMSO (control treatment cells, final concentration of 0,002%), ii) culture medium containing 10% FBS and 75 μ M of 4F-GalNAc for COLO205 WT and Δ C1GalT1 cells, or 150 μ M of 4F-GalNAc for KATOIII cells, during 48 h in a humidified incubator (37°C, 5% CO₂). Then, 2 mL of complete culture medium (media with 10% FBS) was added to the lower part of the insert. After incubation, non-invading cells in the upper part of the insert were carefully removed with a cotton swab, invasion chambers washed with PBS and lower membrane penetrated cells fixed with methanol. Membranes were removed from the inserts and mounted in a microscope glass slide using Vectashield with DAPI (Vector labs). Two independent assays were performed, and cells were seeded in duplicate for each condition. Invading cells were counted under a fluorescence microscope (Carl Zeiss), and measurement was done by counting cells, using ImageJ software, from four random fields at a magnification of 100 $\times$. Results are presented as means $\pm$ SEM for each sample, and the number of invading cells were compared within the different conditions.

E-Selectin binding assay

Alexa 488 anti-Human IgG antibody (dilution 1:500, ThermoFisher) was pre-complexed with a recombinant human E-Selectin (1 μ g/ml; R&D Systems) in TSM buffer (bioWORLD) for 40 min on ice. A total of 2×10^5 cells were incubated with 100 μ l of the complex (or with the Alexa 488 anti-Human antibody as control) for 1h on ice. Cells were washed with TSM buffer and analyzed on a FACS Fortessa Flow cytometer (BDBioscience) and FlowJo software of analysis.

Proteome Profiler Assay

Human Phospho-kinase Antibody array kit (ARY001B, R&D systems) was used to analyze the phosphorylation profiles of several receptor tyrosine kinases (RTK) in 4F-GalNAc treated (75 μ M for COLO205 WT and Δ C1GalT1 cells, and 150 μ M for KATOIII) vs DMSO treated for 48 h. Cells were washed with ice-cold PBS buffer, lysed using lysis buffer provided with the array kit with added protease and phosphate inhibitors, and cells were scrapped and collected into Eppendorf tubes. After centrifugation, the supernatant was collected, and total protein concentrations were determined by BCA assay (Pierce). A total of 500 μ g (COLO205) or 250 μ g (KATOIII) of protein were incubated in the nitrocellulose membranes provided with the kit and were processed and developed using the manufacturer's instructions.

***In vivo* F-MAs testing using a xenograft mice model**

COLO205-LUC+ cells were previously established [28] and used to test 4F-GalNAc treatment. Approximately 2.5×10^5 cells were injected subcutaneously into the dorsum of female N:NIH(s)II:nu/nu nude mice, aged 8–10 weeks and weighing approximately 20 g (i3S animal facility, Portugal). When the tumor reached between 50–100 mm³ volume size, the COLO205-LUC+ xenograft mice were randomized into 3 groups (n = 5 to 6 mice per group) and treated subcutaneously near the tumor, with 100 μ L of saline solution/PBS (control group), 4F-GalNAc or 3F_{ax}-Neu5Ac (both 20 mg/kg body weight) three times per week. *In vivo* luciferase luminescence imaging was performed using the IVIS Spectrum CT (PerkinElmer) at day 1, 7, 14, and 21 of treatment. Animals were anesthetized with 5% (V/V) isoflurane for induction and 1–2% (V/V) for IVIS acquisition. During the treatment period, the tumor volume was measured every other day with a caliper and calculated as TV = (a x b x c) mm³ (Longest axis - a, shortest axis -b, and thickness - c). Animals were sacrificed, and the tumor and organs were collected on day 21 of treatment. All animal assays were conducted in strict conformity with the guidance of the European Union Directive 2010/63/EU, following a protocol previously authorized by the i3S Animal Ethics Committee and the Competent Authority in Portugal (Direção-Geral de Alimentação e Veterinária (DGAV)) (reference 2021_05) regarding the humane endpoints, appropriate husbandry and protection of experimental animals. Mice were bred, cared and fed *ad libidum* at the i3S Animal Facility (Porto, Portugal).

Statistical Analysis

Data was analyzed with GraphPad Prism 8.0 software (CA, USA). Quantitative data were expressed as the mean $\pm$ SEM of three biological replicates. The level of significance was assessed using Student's t-test and one or two-way ANOVA. p - values of <0.05 were considered significant.

Results

4F-GalNAc impacts the biosynthesis of cancer associated O-glycans

To test the capacity of distinct fluorinated GalNAc, 3F- 4F- and 6F-GalNAc (Fig 1A), as inhibitors of cancer-associated O-glycans, we started by assessing its efficacy towards SLeX-positive GIC cells. COLO205 cell line was selected as a model, as it is highly positive for this glycan in glycoproteins [8]. Moreover, SLeX expression is primarily in O-glycans as treatment with PNGase F did not affect this glycan detection (Fig 1B). Among the three tested analogs, only 4F-GalNAc consistently reduced the detection of SLeX in a concentration and time-dependent manner (Fig 1C). This was statistically significant after 24 h of treatment with 20–50% reduction in SLeX immunodetection, depending on the tested concentration. This reduction level was more pronounced at longer time points, reaching an inhibition of 80–90% at 72 h. No significant changes were observed in cells treated with 3F-GalNAc, and only a significant reduction at 72 h was seen in cells treated with 18.75 μ M of 6F-GalNAc (Fig 1C).

To further validate this result, we selected two other SLeX-positive GIC cell lines, KATOIII and LS174T, which express SLeX at different levels in distinct glycoconjugates. KATOIII cells have an expression of SLeX in O-glycans, although with less intensity when compared to COLO205 cells, while in LS174T cells, SLeX is primarily expressed on lipids [8,29] with residual expression in *N*- and *O*- glycans (Fig 1D). As a result, 4F-GalNAc inhibited SLeX expression in KATOIII cells, although at later time-points compared to COLO205 WT cells. The inhibition became significant only after 48 h, reaching around a 60% reduction in SLeX immunodetection at 72 h of treatment (Fig 1E). Regarding LS174T, only a minor inhibitory effect (no more than 20%), that did not reach statistical significance, was observed in LS174T cells (Fig 1F) consistent with its major expression in lipids [8,29].

To avoid the natural variability of O-glycans present in WT cells and to reinforce the efficacy of 4F-GalNAc towards O-glycosylation, we generated a C1GalT1 gene knockout (KO) (Δ C1GalT1) in the GIC cell lines positive for ST6GalNAc1 expression (Fig S1A) COLO205 and MKN45. C1GalT1 is responsible for the extension of initial GalNAc to Core 1 (Fig 1G), and its KO promotes the expression of the truncated O-glycans Tn and STn (Fig S1B and C). We observed that Δ C1GalT1 abolished the expression of SLeX in COLO205, reinforcing that this glycan is attached primarily to O-glycans (Fig 1H), as previously mentioned. By testing the distinct fluorinated GalNAcs, we observed that only 4F-GalNAc significantly reduced STn immunodetection in COLO205 Δ C1GalT1 (Fig 1I). Similar to the effect on WT cells, a significant reduction was observed at 24 h, becoming more pronounced over time, reaching a maximum of 60% reduction of STn immunodetection at 72 h. This reduction was also confirmed by western blot analysis (Fig S1D). On the contrary, only lower concentrations of 3F-GalNAc caused a minor reduction of STn at 24 h, and this trend was

not maintained at longer time points. Furthermore, no reduction in STn immunodetection was seen with 6F-GalNAc (Fig 1I). The capacity of 4F-GalNAc to impact O-glycosylation was also validated by a decrease in Tn antigen detection by flow cytometry, similarly to the pattern seen with STn (Fig S1E). Using MKN45 Δ C1GalT1 cell model, which also expresses the truncated O-glycans Tn and STn (Fig S1F and G), we could also observe a reduction in STn immunodetection with 4F-GalNAc treatment (Fig S1H).

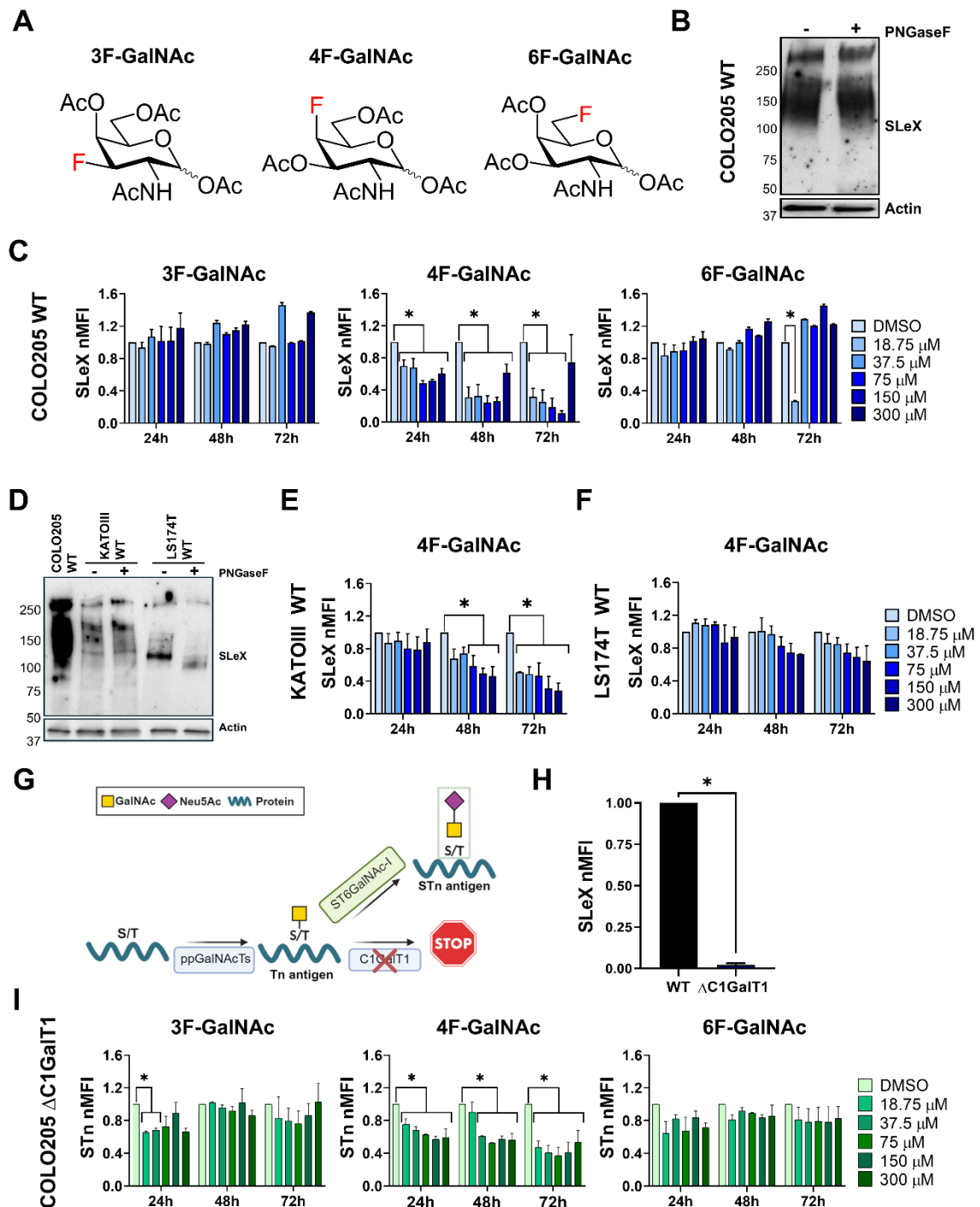


Figure 1: Impact of distinct fluorinated GalNAc in cancer-associated O-glycan biosynthesis A) Chemical structure of the three distinct fluorinated GalNAc tested in this study: 3F-GalNAc, 4F-GalNAc and 6F-GalNAc.

B) Western blot analysis of SLeX showing its primarily presence in O-glycans in COLO205 cells. **C)** Flow cytometry analysis of SLeX immunodetection in COLO205 WT cells after treatment with distinct fluorinated GalNAc in a concentration and time-dependent manner. Results show the capacity of 4F-GalNAc is reducing SLeX immunodetection with no significative alterations observed with 3F and 6F-GalNAc treatment. **D)** Western blot analysis of SLeX in COLO205, KATOIII and LS174T WT cells treated with and without PNGaseF, showing its differential presence in *N*- and O-glycans. **E)** Flow cytometry analysis of SLeX immunodetection in KATOIII cells after treatment with 4F-GalNAc showing around 60% reduction of SLeX expression. **F)** Flow cytometry analysis of SLeX immunodetection in LS174T cells after treatment with 4F-GalNAc showing no significant reduction of SLeX expression. **G)** Schematic representation of the glycoengineered strategy to establish Tn/STn expressing GIC cells. **H)** Flow cytometry analysis of SLeX immunodetection in COLO205 Δ C1GalT1 cells showing its presence mainly in O-Glycans. **I)** Flow cytometry analysis of STn immunodetection in COLO205 Δ C1GalT1 cells after treatment with distinct fluorinated GalNAc in a concentration and time-dependent manner. Results show a reduction in STn immunodetection in cells treated only with 4F-GalNAc. Results are presented as mean $\pm$ SEM of three independent experiments. * $p < 0.01$ vs. the DMSO-treated group. nMFI: Median fluorescent intensity normalized to DMSO.

To decipher the main alteration within cells treated with 4F-GalNAc, we performed RNAseq and analyzed altered genes according to molecular networks. Interestingly, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of COLO205 WT cells treated with 4F-GalNAc vs DMSO revealed significant alterations in pathways associated with the biosynthesis of both nucleotide sugar donors and *N*-glycan (Fig 2). Similarly, the same analysis in COLO205 Δ C1GalT1 and KATOIII WT cells showed alterations in the pathways associated with the biosynthesis of *N*-glycans (Figs S2C and F). Regarding the alterations found in the nucleotide sugar metabolism pathway in both COLO205 WT and COLO205 Δ C1GalT1 cells, we mainly observed altered genes associated with the metabolism and biosynthesis of the UDP-GalNAc and UDP-GlcNAc activated donors. Furthermore, 4F-GalNAc treatment had an impact on the expression of some genes involved in the synthesis of sialic acid (Neu5Ac) and mannose (Man) sugar donors, CMP-Neu5Ac and GDP-Man respectively (Fig S2A and D). For KATOIII, similar alterations were observed in genes comprising the metabolic pathway of GalNAc/ GlcNAc and Man, however, no genes related to sialic acid CMP conversion seem affected (Fig S2G). Additionally, we observed alteration (mainly upregulation) of several genes related to *N*-glycan biosynthesis. This is essentially observed in the COLO205 WT and COLO205 Δ C1GalT1 (Fig S2B and E) treated cells with less extent for KATOIII WT cells (Fig S2H).

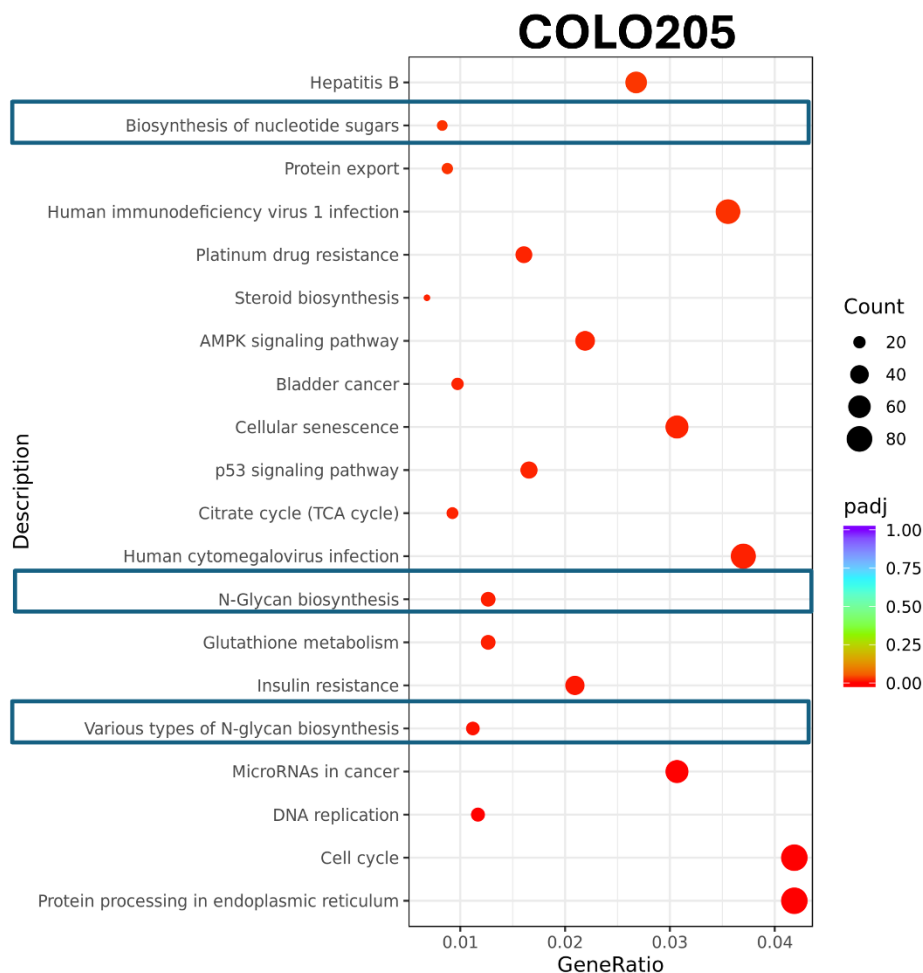


Figure 2: Transcriptomic analysis by RNA sequencing comparing 4F-GalNAc and DMSO-treated COLO205 WT cells. KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis showing alterations in the metabolic pathways of sugar donors and in the biosynthesis of N-glycans.

4F-GalNAc treatment impairs malignant features of GIC cells

To infer the impact of 4F-GalNAc in malignant characteristics of cancer cells, we assessed different biological parameters considering treatment with 75 μ M in COLO205 WT and COLO205 Δ C1GalT1 cells, and 150 μ M in KATOIII cells all for 48 h. The treatment, although not statistically significant, showed a tendency to reduce the metabolic rate of COLO205 cells (Fig 3A and Fig S3A). However, this trend was not observed in KATOIII cells (Fig 3A). On the contrary, 4F-GalNAc treatment significantly inhibited migration and invasion capacity in all tested cell lines (Fig 3B and C and Fig S3B and C).

The binding of SLeX and SLeA expressed in cancer cells to E-Selectin present in endothelial cells is an important property for cancer cell dissemination and metastasis development [30]. To assess the impact of 4F-GalNAc on E-selectin binding, we first evaluated the expression of SLeA in tested cells. COLO205 WT cells exhibit high expression of SLeA, which was significantly reduced by C1GalT1 KO (around 75%), pointing to its

presence in other glycans rather than core 1 and 2 terminal structures (Fig S3D). Additionally, Δ C1GalT1 led to a reduction in E-selectin binding, ranging from 60–70% (Fig S3E). Treatment with 4F-GalNAc resulted in a reduction of SLeA by approximately 60% in WT cells and nearly 90% in Δ C1GalT1 cells (Fig 3D and Fig S3F). Hence, we also observe a reduction in E-Selectin binding by approximately 50% in COLO205 WT cells and 70% in Δ C1GalT1 cells (Fig 3E and Fig S3G).

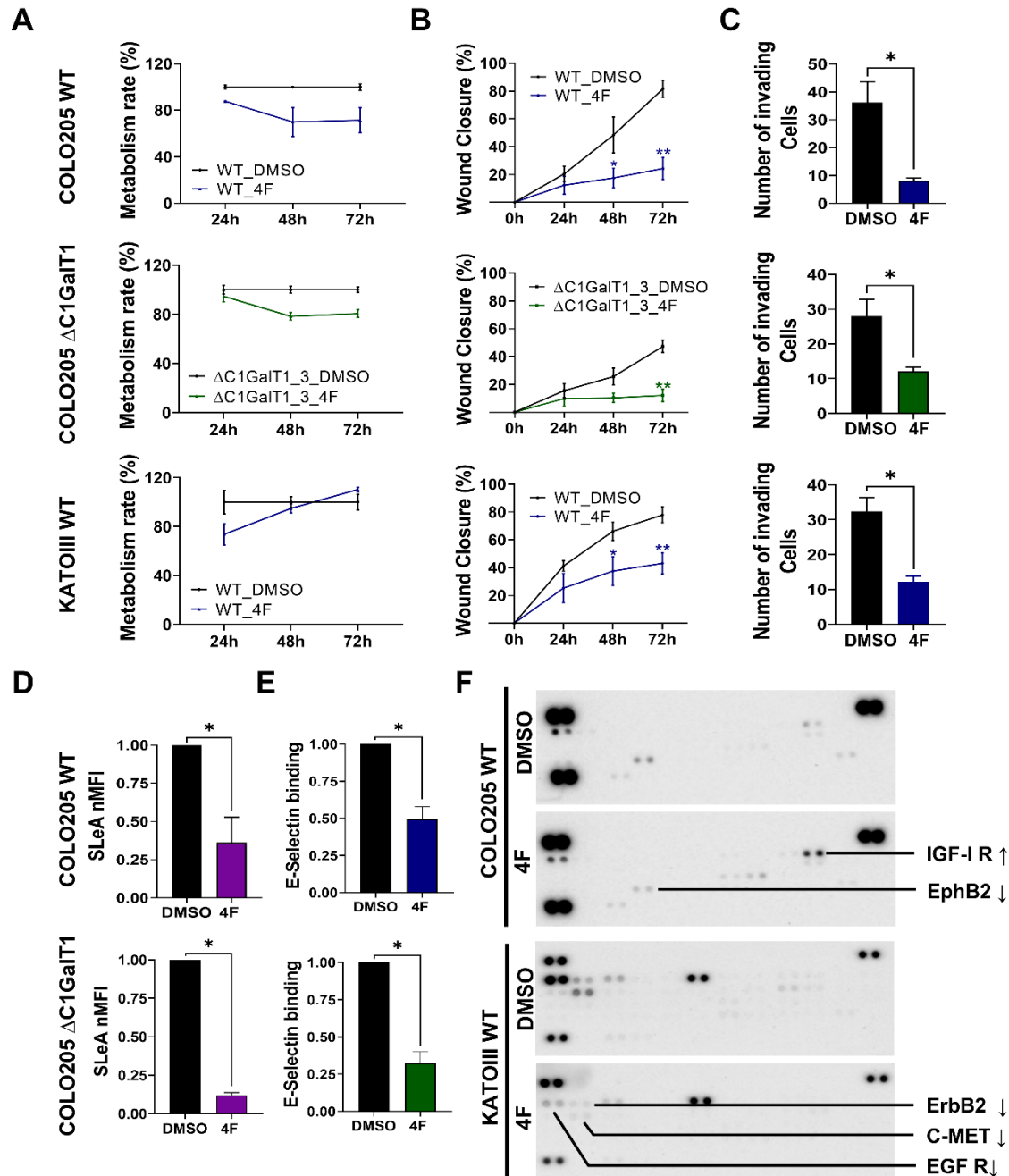


Figure 3: Biological impact of 4F-GalNAc treatment in GIC cells. **A)** Cell metabolism (evaluated by MTT assay) in COLO205 WT, COLO205 Δ C1GalT1 clone 3, and in KATOIII WT during 72h of treatment with 4F-GalNAc showing some differences between cell lines. **B)** Wound healing assay of COLO205 WT, COLO205 Δ C1GalT1 clone 3 and KATOIII WT cancer cells treated with 4F-GalNAc during 72h showing a reduction in cell motility in all tested cell lines. **C)** Invasive capacity of COLO205 WT, COLO205 Δ C1GalT1 clone

3 and KATOIII WT cancer cells treated with 4FGalNaAc showing a reduction in the invasive capacity in all tested cells. **D)** Flow cytometry analysis of SLeA immunodetection in COLO205 and COLO205ΔC1GalT1 clone 3 showing its expression besides core1/2 structures. **E)** E-Selectin binding assay in COLO205 WT and COLO205ΔC1GalT1 clone 3 cells after treatment with 4F-GalNAc showing a reduction in the binding capacity of cells to E-Selectin after treatment with 4F-GalNAc. Results are presented as mean ± SEM of three independent experiments. * $p < 0.01$ 4F-GalNAc-treated vs DMSO-treated group. **F)** Phospho-RTK array of COLO205 WT and in KATOIII WT cells comparing DMSO and 4F-GalNAc-treated cells. Results show that 4F-GalNAc treatment led to an increase in the activation of IGF-I R and a reduction in the activation of EphB2 in COLO205 WT cells, whereas in KATOIII WT cells it is observed a reduction in the activation of ErbB2, C-MET and EGFR.

To assess the impact of 4F-GalNAc on cell surface receptor activation and to identify possible targeted signaling pathways, a receptor tyrosine kinase (RTK) array was performed on both COLO205 and KATOIII WT cells. In general, the 4F-GalNAc treatment led to distinct alterations in RTK activation in the different cell lines. In detail, in COLO205 the treatment led to a significant increase in the Insulin-like growth factor 1 (IGF-I) receptor activation and a moderate decrease in Ephrin type-B receptor 2 (EphB2) activation. On the other hand, KATOIII presented a reduction in the activation of several RTKs such as hepatocyte growth factor receptor (HGFR/ c-MET), epidermal growth factor receptor (EGFR), and Human Epidermal Growth Factor Receptor 2 (ErbB2) (Fig 3F).

4F-GalNAc treatment impacts tumor growth in mice

To assess if 4F-GalNAc also impacts SLeX expression and cancer cell malignancy *in vivo*, we used a COLO205-LUC+ xenograft mouse model. We subcutaneously injected 2.5×10^5 cells on the dorso of each mouse and once the tumor reached 50–10mm³, mice were divided into 3 groups: PBS, 4F-GalNAc, and 3F_{ax}-Neu5Ac (Fig 4A). 3F_{ax}-Neu5Ac (Fig S4A) was used as a control as several studies have demonstrated its efficacy in reducing tumor growth [12,31,32], and we show its capacity in reducing *in vitro* SLeX immunodetection in COLO205 WT cells (Fig S4B). Subcutaneous administration, near the tumor site, of both 4F-GalNAc and 3F_{ax}-Neu5Ac significantly inhibited tumor growth compared to the PBS-treated group, resulting in smaller tumors by day 21 (Fig 4B and C). Although there were no statistically significant differences between both analogs, 4F-GalNAc showed a tendency to be more efficient in inhibiting tumor growth of COLO205 xenografts when compared to 3F_{ax}-Neu5Ac (Fig 4B).

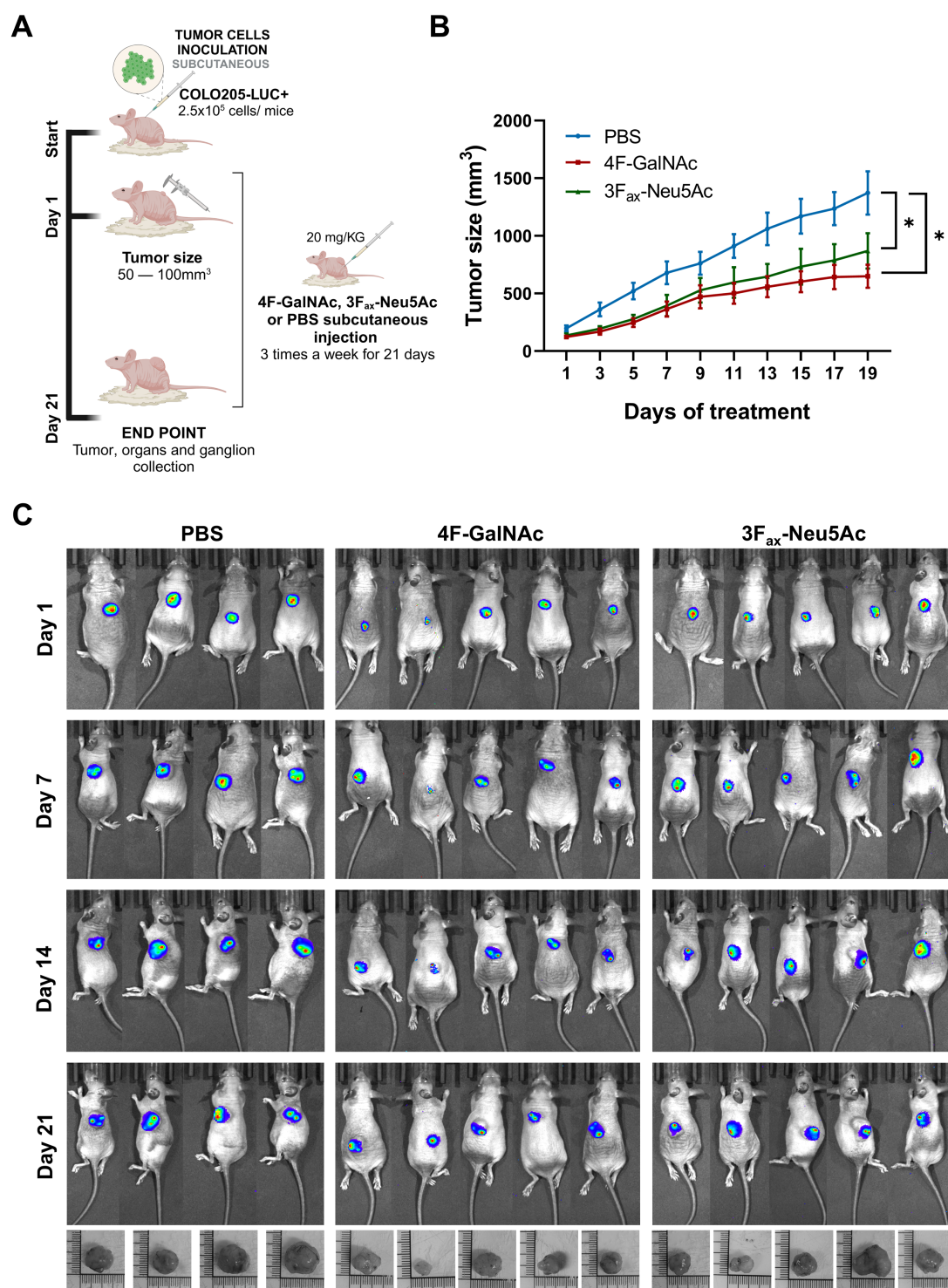


Figure 4: *In vivo* biological effect of 4F-GalNAc treatment using a xenograft mouse model. A) Schematic representation of the xenograft mouse model experiment design. **B)** Tumor growth rate along time showing a reduced tumor size in 4F-GalNAc-treated mice. Results are presented as mean $\pm$ SEM. * $P < 0.01$ vs. PBS-treated group. **C)** Representative images of *in vivo* tumor growth obtained by measuring luciferase activity throughout time, showing the reduced tumor growth in 4F-GalNAc-treated mice.

Discussion

GIC, mainly CRC and GC, rank at the top of the deadliest cancers, and the currently available therapies present several limitations, which emphasize the urgent need for new treatment options targeting emergent cancer biomarkers. Aberrant expression of glycan antigens is a feature in cancer, and glycan structures such as Tn and STn truncated O-glycans and SLeX and SLeA sialylated terminal structures have been linked to a more aggressive behavior of cancer cells [4,5]. Thus, options targeting cancer-associated glycan antigens and their biosynthesis are currently being tested in several studies and, even, in clinical trials [13,15,33-35]. The use of F-MAs as glycosylation metabolic inhibitors represents a clever and effective strategy for developing an effective therapy. While fucose and sialic acid fluorinated analogs are largely explored as cancer therapeutics and are very specific in inhibiting general fucosylation and sialylation [11,13], HexNAc analogs, such as F-GalNAc are poorly explored.

Therefore, in this study, we evaluated the impact of three distinct fluorinated GalNAc analogs, where the difference between them is the position of the added fluorine atom at carbon 3, 4, or 6 forming 3F-, 4F-, and 6F-GalNAc, respectively. Using distinct GIC cell models with diverse glycosylation backgrounds, we demonstrated that 4F-GalNAc can specifically reduce the immunodetection of O-linked SLeX. Moreover, in established C1GalT1 KO cell models, resulting in cells with high expression of short and easily detectable Tn and STn O-glycan antigens, 4F-GalNAc also led to a significant reduction in their immunodetection. These results are in line with the already described effect of 4F-GalNAc on SLeX expression and O-glycans biosynthesis [20,21]. Interestingly, also for GlcNAc analogs, 4F-GlcNAc has been described as a highly effective inhibitor of glycosylation [16,18,20], reinforcing the importance of the position of the fluorine atom at carbon 4 for effective HexNAc inhibitory capacity [11]. Following this line, no consistent inhibition was observed with 3F- and 6F-GalNAc. For 3F-GalNAc, a minor reduction in STn expression was observed only at 24h of treatment at few tested concentrations, a result that was not consistent with longer incubation periods. This led us to conclude that 3F-GalNAc is not a potent inhibitor of O-glycans. In fact, in a study comparing the toxicity of several HexNAc analogs, 3F-GalNAc presented less toxicity in different cell lines [27], highlighting its almost inert effect on cells. Regarding 6F-GalNAc, we observed a 70% inhibition of SLeX detection at 72h, with the lowest concentration. This could suggest that this analog could be more efficient at lower concentrations, however, it is strange that it only presents an inhibitory phenotype after 72h whereas 4F-GalNAc showed to be very efficient at 24h. To date, only one study showed the efficacy of 6F-GalNAc (50 μ M) in reducing the expression of GAGs, *N*-acetylglucosamine (GlcNAc), and core 1 structures after 72h treatment [20]. In our study, we focused on specific O-glycans structures, SLeX and STn, and using narrow

concentrations (37.5 and 75 μ M) for 72 h, we did not observe significant alterations in their immunodetection. This could be justified by the different cancer cells used as models, which may present distinct metabolic activities. Thus, this reinforces that concentration and time points need to be optimized for different cell lines.

Further, transcriptomic analysis through RNAseq showed alteration in genes involved in the sugar metabolism pathway, mainly in genes involved in the conversion of GalNAc, GlcNAc, Neu5Ac, and Man monosaccharides into their activated forms. This raises the question of the mechanism of action behind the use of F-MAs within cells. It is well established that F-MAs, after entering the cells, are converted into their corresponding fluorinated sugar donors. Both fluorinated Fuc and Neu5Ac are thought to act as inhibitors of *de novo* synthesis of the corresponding natural sugar donor through a negative feedback loop [11]. Similarly, 4F-GlcNAc has been described to be metabolized to UDP-4F-GlcNAc, leading to decreased levels of the natural nucleotide sugars UDP-GlcNAc and UDP-GalNAc [18,36]. None of these studies were able to show the analog incorporation within glycan biosynthetic pathways. However, in the case of 4F-GalNAc, Marathe and colleagues showed that this analog can be incorporated into glycan structures on multiple cellular glycoproteins [21]. A small degree of incorporation was described for some fluorinated Fuc derivatives [13,37] and other non-fluorinated analogs [38], including GalNAc analogs, such as GalNAz [22]. However, for 4F-GlcNAc and 6F-GalNAc, no incorporation was observed [18,20]. In our study, preliminary mass spectrometry structural analysis of released O-glycans (not shown) pointed to no incorporation of 4F-GalNAc in glycan structures. In addition, we have observed a reduction of Tn antigen detection, similar to STn, and despite we cannot discard that fluorine can interfere with antibody recognition, it is unlikely that this is the main mechanism behind 4F-GalNAc inhibitory effects. In fact, other studies suggested that 4F-GalNAc acts by inhibiting the production of endogenous UDP-GalNAc/GlcNAc and, also, CMP-Neu5Ac and GDP-Man [23,39]. This corroborates the observed changes in the sugar metabolism pathway through KEGG analysis of RNAseq data in our study. Nevertheless, confirmation studies such as the evaluation of the natural sugar donor levels after treatment, and enzymatic assays are required.

In addition to sugar donor metabolism, RNAseq analysis showed upregulation of several genes involved in the *N*-glycosylation biosynthesis pathway. Effects in *N*-glycosylation are not surprising, as previous studies showed *N*-glycan changing after 4F-GalNAc treatment [20,21,39]. Alterations of the sugar donor levels such as UDP-GlcNAc and GDP-Man could be responsible for the change observed. Furthermore, as this analog impairs O-glycosylation elongation, cells can alter or even increase the synthesis of terminal *N*-glycans as a compensatory mechanism. Thus, deep characterization of *N*-glycan

structures in 4F-GalNAc treated cells must be performed to better assign 4F-GalNAc impact on general glycosylation.

4F-GalNAc treatment consequently had a significant impact on the biological behavior of cancer cells. In particular, we saw a huge reduction in the migration and invasion capacity of cancer cells, important features in the process of metastasis. Many reports showed the important role and clinical association between SLeX and SLeA expression and an invasive tumor phenotype in several tumors [7,8]. For instance, these glycans expressed in cancer cells are known to interact with E-selectin present in endothelial cells facilitating cancer cells spread in the bloodstream to distant sites [30]. Thus, this result was expected since 4F-GalNAc treatment led to a huge impact on SLeX and SLeA biosynthesis, and also on E-selectin binding capacity.

Furthermore, 4F-GalNAc affected the cell surface RTKs activation. These receptors are transmembrane glycoproteins and consequently, alteration in their normal glycosylation pattern can disturb their function [40,41]. On top of this, many of these receptors' activation also depends on the interaction with other cell surface glycoconjugates that can be affected by 4F-GalNAc treatment. Specifically, in COLO205-treated cells, we observed an increase in IGF-I receptor activation. This highly glycosylated receptor is commonly found overexpressed and associated with more malignant characteristics of cancer cells, such as CRC cells [42]. Additionally, it was shown that changes in *N*-glycosylation could affect IGF-I activation and expression at the cell surface [43], and overexpression of the enzyme pp-GalNAcT2, responsible for the initiation of O-GalNAc glycosylation, inhibits IGF-I signaling in neuroblastoma [44]. These reports support our finding that alteration in cell surface glycosylation through 4F-GalNAc impacted IGF-I receptor activation, however validation is needed. Also, in COLO205 cells, we observed a reduction in EphB2 activation upon 4F-GalNAc treatment. The overexpression of this gene can be found in many tumors, including CRC, and is linked to cell motility and invasion, elevated metastatic potential, and poor prognosis for cancer patients [45]. Similarly, glycosylation plays a role in this receptor family activation and interaction, affecting the proliferation of cancer cells [46]. Regarding KATOIII cells, we mainly observed a general decrease in RTK activation with 4F-GalNAc treatment. EGFR, ErbB2, and c-MET are well-described as overexpressed and differently activated in GC, and their expression is associated with an increased recurrence of the disease [47]. Moreover, these receptors have an important role in the aggressive phenotype of cancer cells, with glycosylation playing an important role. For example, the loss of core fucosylation leads to reduced biological function of EGFR [48]. Moreover, overexpression of pp-GalNAcT 2 and 6 leads to EGFR activation and enhances the malignant properties, such as migration and invasion, of oral [49] and ovarian cancer cells [50]. Also, our group showed that induced expression of SLeX through overexpression of ST3GalIV leads to increased activation of c-

MET and a more aggressive phenotype in CG cells [7]. In liver cancer, C1GALT1 overexpression modulated c-MET glycosylation, consequently increasing its activation and enhancing tumor growth [51]. In line with this, we showed that the inhibition of O-linked glycans, such as SLeX, by 4F-GalNAc, led to decrease activation of c-MET. Furthermore, our group showed a correlation between the expression of SLeA glycoforms in ErbB2 and the correlation between ErbB2 expression and FUT3, an enzyme involved in SLeA biosynthesis, in GC patients [52]. Hence, we conclude that the direct effect of 4F-GalNAc treatment in cancer cells glycosylation remodeling is the main responsible for the alteration of RTKs activation and, consequently, in the biological impact observed in treated cells.

Finally, *in vivo* administration of 4F-GalNAc has an impact on tumor growth using a xenograft mouse model. In our study, we used as control the vastly tested 3F_{ax}-Neu5Ac [12,31,32], and similar inhibitory effects on tumor growth were observed using both analogs. In fact, 4F-GalNAc tended to be more efficient than 3F_{ax}-Neu5Ac in inhibiting tumor growth. Multiple concerns arise due to the toxicity of these analogs as, for instance, 3F_{ax}-Neu5Ac is known to cause renal failure in mice [53]. Thus, a delivery system was further used to safely deliver 3F_{ax}-Neu5Ac to cancer cells [54], as well as a structural modification that led to its photo-induced activation [55]. Nevertheless, the only report on *in vivo* testing of 4F-GalNAc showed that intravenous injection of higher doses did not cause toxicity to mice [21]. Thus, we can hypothesize that fluorinated GalNAc could have a safer profile, however, the time window of testing was relatively short. In our study, we did not observe any toxicity or signs of distress in the mice. Nonetheless, we performed a local injection of the drugs for a period of 21 days. Thus, specific studies are needed to clarify the pharmacokinetics and toxicity of 4F-GalNAc.

Conclusion

This study underscores the potential of 4F-GalNAc as a promising therapeutic agent for targeting aberrant glycosylation in cancer. By interfering with the biosynthesis of cancer-associated O-glycans, 4F-GalNAc impacts RTKs activation and alters cancer cell biological behavior, leading to reduced migration, invasion and E-selectin binding. Its efficacy in reducing tumor growth, using an *in vivo* model, supports its potential as a novel approach for developing a new cancer therapy.

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Contributions

CG and CR supervised the study. AFC and CG designed the experiments. VM and JK performed the synthesis of the F-MAs. ES, CG and AFC establish the KO cell models. AFC, ES, AT, RA, IFR and CG performed experiments and analyzed the data. AFC and AT performed the *in vivo* experiments. AFC and CG wrote the original paper. All authors critically revised the paper.

Conflicts of Interest

The authors declare no conflict of interest.

References

- [1] Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024 May-Jun;74(3):229-263.
- [2] Costa AF, Campos D, Reis CA, et al. Targeting Glycosylation: A New Road for Cancer Drug Discovery. *Trends Cancer.* 2020 Sep;6(9):757-766.

- [3] Anand U, Dey A, Chandel AKS, et al. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. *Genes Dis.* 2023 Jul;10(4):1367-1401.
- [4] Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer.* 2015 Sep;15(9):540-55.
- [5] Mereiter S, Balmana M, Campos D, et al. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? *Cancer Cell.* 2019 Jul 8;36(1):6-16.
- [6] Munkley J. The Role of Sialyl-Tn in Cancer. *Int J Mol Sci.* 2016 Feb 24;17(3):275.
- [7] Gomes C, Osorio H, Pinto MT, et al. Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS One.* 2013 Jun;8(6):e66737.
- [8] Costa AF, Senra E, Faria-Ramos I, et al. ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and associates with cancer cell motility. *Glycoconj J.* 2023 Aug;40(4):421-433.
- [9] Freitas D, Campos D, Gomes J, et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. *EBioMedicine.* 2019 Feb;40:349-362.
- [10] Rossing E, Pijnenborg JFA, Boltje TJ. Chemical tools to track and perturb the expression of sialic acid and fucose monosaccharides. *Chem Commun (Camb).* 2022 Nov 1;58(87):12139-12150.
- [11] Rillahan CD, Antonopoulos A, Lefort CT, et al. Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. *Nat Chem Biol.* 2012 Jul;8(7):661-8.
- [12] Bull C, Boltje TJ, Wassink M, et al. Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. *Mol Cancer Ther.* 2013 Oct;12(10):1935-46.
- [13] Orozco-Moreno M, Visser EA, Hodgson K, et al. Targeting aberrant sialylation and fucosylation in prostate cancer cells using potent metabolic inhibitors. *Glycobiology.* 2023 Dec 30;33(12):1155-1171.
- [14] Carrascal MA, Silva M, Ramalho JS, et al. Inhibition of fucosylation in human invasive ductal carcinoma reduces E-selectin ligand expression, cell proliferation, and ERK1/2 and p38 MAPK activation. *Mol Oncol.* 2018 May;12(5):579-593.
- [15] Do KT, Chow LQM, Reckamp K, et al. First-In-Human, First-In-Class, Phase I Trial of the Fucosylation Inhibitor SGN-2FF in Patients with Advanced Solid Tumors. *Oncologist.* 2021 Nov;26(11):925-e1918.
- [16] Woynarowska B, Skrincosky DM, Haag A, et al. Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. *J Biol Chem.* 1994 Sep 9;269(36):22797-803.

- [17] Woynarowska B, Dimitroff CJ, Sharma M, et al. Inhibition of human HT-29 colon carcinoma cell adhesion by a 4-fluoro-glucosamine analogue. *Glycoconj J*. 1996 Aug;13(4):663-74.
- [18] Barthel SR, Antonopoulos A, Cedeno-Laurent F, et al. Peracetylated 4-fluoro-glucosamine reduces the content and repertoire of N- and O-glycans without direct incorporation. *J Biol Chem*. 2011 Jun 17;286(24):21717-31.
- [19] Cedeno-Laurent F, Opperman MJ, Barthel SR, et al. Metabolic inhibition of galectin-1-binding carbohydrates accentuates antitumor immunity. *J Invest Dermatol*. 2012 Feb;132(2):410-20.
- [20] van Wijk XM, Lawrence R, Thijssen VL, et al. A common sugar-nucleotide-mediated mechanism of inhibition of (glycosamino)glycan biosynthesis, as evidenced by 6F-GalNAc (Ac3). *FASEB J*. 2015 Jul;29(7):2993-3002.
- [21] Marathe DD, Buffone A, Jr., Chandrasekaran EV, et al. Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins. *Blood*. 2010 Feb 11;115(6):1303-12.
- [22] Pouilly S, Piller V, Piller F. Metabolic glycoengineering through the mammalian GalNAc salvage pathway. *FEBS J*. 2012 Feb;279(4):586-98.
- [23] Jackson EG, Cutolo G, Yang B, et al. 4-Deoxy-4-fluoro-GalNAz (4FGalNAz) Is a Metabolic Chemical Reporter of O-GlcNAc Modifications, Highlighting the Notable Substrate Flexibility of O-GlcNAc Transferase. *ACS Chem Biol*. 2022 Jan 21;17(1):159-170.
- [24] Narimatsu Y, Joshi HJ, Yang Z, et al. A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome. *Glycobiology*. 2018 May 1;28(5):295-305.
- [25] Lonowski LA, Narimatsu Y, Riaz A, et al. Genome editing using FACS enrichment of nuclease-expressing cells and indel detection by amplicon analysis. *Nat Protoc*. 2017 Mar;12(3):581-603.
- [26] Brinkman EK, Chen T, Amendola M, et al. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res*. 2014 Dec 16;42(22):e168.
- [27] Hornik S, Cervenková Stastná L, Curinová P, et al. Synthesis and in vitro cytotoxicity of acetylated 3-fluoro, 4-fluoro and 3,4-difluoro analogs of D-glucosamine and D-galactosamine. *Beilstein J Org Chem*. 2016;12:750-9.
- [28] Costa AF, Senra E, Campos D, et al. Monensin as potential drug for treatment of SLeX-positive tumors. *medRxiv*. 2024 Mar:2024.03.11.24304048.

- [29] Wang D, Madunic K, Zhang T, et al. High Diversity of Glycosphingolipid Glycans of Colorectal Cancer Cell Lines Reflects the Cellular Differentiation Phenotype. *Mol Cell Proteomics*. 2022 Jun;21(6):100239.
- [30] Trinchera M, Aronica A, Dall'Olio F. Selectin Ligands Sialyl-Lewis a and Sialyl-Lewis x in Gastrointestinal Cancers. *Biology (Basel)*. 2017 Feb 23;6(1).
- [31] Bull C, Boltje TJ, Balneger N, et al. Sialic Acid Blockade Suppresses Tumor Growth by Enhancing T-cell-Mediated Tumor Immunity. *Cancer Res*. 2018 Jul 1;78(13):3574-3588.
- [32] Miro L, Lopez J, Guerrero PE, et al. Sialyltransferase Inhibitor Ac(5)3F(ax)Neu5Ac Reverts the Malignant Phenotype of Pancreatic Cancer Cells, and Reduces Tumor Volume and Favors T-Cell Infiltrates in Mice. *Cancers (Basel)*. 2022 Dec 12;14(24).
- [33] Martin KC, Tricoli J, Corzana F, et al. Fucosyltransferase-specific inhibition via next generation of fucose mimetics. *Chem Commun (Camb)*. 2021 Jan 28;57(9):1145-1148.
- [34] Morla S, Ravikumar O, O'Hara C, et al. Designing Synthetic, Sulfated Glycosaminoglycan Mimetics That Are Orally Bioavailable and Exhibiting In Vivo Anticancer Activity. *J Med Chem*. 2023 Jan 26;66(2):1321-1338.
- [35] Wang X, Zhang Y, Lin H, et al. Alpha2,3-sialyltransferase III knockdown sensitized ovarian cancer cells to cisplatin-induced apoptosis. *Biochem Biophys Res Commun*. 2017 Jan 22;482(4):758-763.
- [36] Nishimura S, Hato M, Hyugaji S, et al. Glycomics for drug discovery: metabolic perturbation in androgen-independent prostate cancer cells induced by unnatural hexosamine mimics. *Angew Chem Int Ed Engl*. 2012 Apr 2;51(14):3386-90.
- [37] Allen JG, Mujacic M, Frohn MJ, et al. Facile Modulation of Antibody Fucosylation with Small Molecule Fucostatin Inhibitors and Cocrystal Structure with GDP-Mannose 4,6-Dehydratase. *ACS Chem Biol*. 2016 Oct 21;11(10):2734-2743.
- [38] Zimmermann M, Nguyen M, Schultheiss CM, et al. Use of 5-Thio-L-Fucose to modulate binding affinity of therapeutic proteins. *Biotechnol Bioeng*. 2021 May;118(5):1818-1831.
- [39] Del Solar V, Gupta R, Zhou Y, et al. Robustness in glycosylation systems: effect of modified monosaccharides, acceptor decoys and azido sugars on cellular nucleotide-sugar levels and pattern of N-linked glycosylation. *Mol Omics*. 2020 Aug 1;16(4):377-386.
- [40] Ferreira IG, Pucci M, Venturi G, et al. Glycosylation as a Main Regulator of Growth and Death Factor Receptors Signaling. *Int J Mol Sci*. 2018 Feb 15;19(2).

- [41] Lopez-Sambrooks C, Shrimall S, Khodier C, et al. Oligosaccharyltransferase inhibition induces senescence in RTK-driven tumor cells. *Nat Chem Biol.* 2016 Dec;12(12):1023-1030.
- [42] Vigneri PG, Tirro E, Pennisi MS, et al. The Insulin/IGF System in Colorectal Cancer Development and Resistance to Therapy. *Front Oncol.* 2015 Oct;5:230.
- [43] de-Freitas-Junior JCM, Andrade-da-Costa J, Silva MC, et al. Glycans as Regulatory Elements of the Insulin/IGF System: Impact in Cancer Progression. *Int J Mol Sci.* 2017 Sep 7;18(9).
- [44] Ho WL, Chou CH, Jeng YM, et al. GALNT2 suppresses malignant phenotypes through IGF-1 receptor and predicts favorable prognosis in neuroblastoma. *Oncotarget.* 2014 Dec 15;5(23):12247-59.
- [45] Liu W, Yu C, Li J, et al. The Roles of EphB2 in Cancer. *Front Cell Dev Biol.* 2022 Feb;10:788587.
- [46] Xu Y, Robev D, Saha N, et al. The Ephb2 Receptor Uses Homotypic, Head-to-Tail Interactions within Its Ectodomain as an Autoinhibitory Control Mechanism. *Int J Mol Sci.* 2021 Sep 28;22(19).
- [47] Wang J, Wang S, Sun J, et al. Expression of c-MET, EGFR and HER-2 in gastric adenocarcinoma tissue and its relationship with clinicopathological characteristics. *Am J Transl Res.* 2021 Sep;13(9):10856-10862.
- [48] Wang Y, Fukuda T, Isaji T, et al. Loss of alpha1,6-fucosyltransferase inhibits chemical-induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways. *FASEB J.* 2015 Aug;29(8):3217-27.
- [49] Lin MC, Huang MJ, Liu CH, et al. GALNT2 enhances migration and invasion of oral squamous cell carcinoma by regulating EGFR glycosylation and activity. *Oral Oncol.* 2014 May;50(5):478-84.
- [50] Lin TC, Chen ST, Huang MC, et al. GALNT6 expression enhances aggressive phenotypes of ovarian cancer cells by regulating EGFR activity. *Oncotarget.* 2017 Jun 27;8(26):42588-42601.
- [51] Wu YM, Liu CH, Huang MJ, et al. C1GALT1 enhances proliferation of hepatocellular carcinoma cells via modulating MET glycosylation and dimerization. *Cancer Res.* 2013 Sep 1;73(17):5580-90.
- [52] Duarte HO, Balmana M, Mereiter S, et al. Gastric cancer cell glycosylation as a modulator of the ErbB2 oncogenic receptor. *Int J Mol Sci.* 2017 Oct 28;18(11).
- [53] Macauley MS, Arlian BM, Rillahan CD, et al. Systemic blockade of sialylation in mice with a global inhibitor of sialyltransferases. *J Biol Chem.* 2014 Dec 19;289(51):35149-58.

- [54] Bull C, Boltje TJ, van Dinther EA, et al. Targeted delivery of a sialic acid-blocking glycomimetic to cancer cells inhibits metastatic spread. *ACS Nano*. 2015 Jan 27;9(1):733-45.
- [55] Moons SJ, Hornikx D, Aasted MKM, et al. UV light-induced spatial loss of sialic acid capping using a photoactivatable sialyltransferase inhibitor. *RSC Chem Biol*. 2023 Jul 5;4(7):506-511.

Supplementary material for:

4F-GalNAc is a general inhibitor of cancer-associated O-glycans in gastrointestinal tumors

Ana F. Costa, Voltech Hamala, Emanuel Senra, Andreia Teixeira, Rafaela Abrantes, Isabel Faria-Ramos, Celso A. Reis, Jindrich Karban and Catarina Gomes

→ Supplementary text – Materials and Methods

Cell lines and cell culture

NCI-N87, SNU1, SNU638, CACO-2, HT29, HCT15, HCT116, DLD1, RKO, SW48, SW480, SW620 were obtained from the American Type Culture Collection (ATCC). HEK293 (WT and Δ COSMIC +ST6GALNAC1) were kindly provided by Prof. Henrik Clausen. Cells were grown in monolayer culture in 6-well plates (Fisher Scientific) and maintained at 37 °C in an atmosphere of 5% CO₂, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX HEPES medium or Dulbecco's Modified Eagle Medium (DMEM) (all from Biowest), supplemented with 10% fetal bovine serum (FBS) (Biowest).

RNA extraction and reverse transcription

Total RNA was extracted from cellular pellets using the TRI Reagent (Sigma) protocol, according to manufacturer instructions. Isolated RNA was then quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and converted to cDNA by reverse transcription (RT). RT was performed using a mixture containing 3 µg of total RNA, 1 µL of random oligonucleotides primers, 1 µL of 10 mM deoxynucleotide triphosphates (dNTPs), 4 µL of 5X RT buffer, 1 µL of 100 mM dithiothreitol (DTT), 1 µL of RNaseOUT (40 U/µL), 1 µL of SuperScriptR IV reverse transcriptase (200 U/µL) (Invitrogen) and nuclease-free water in a final reaction volume of 20 µL, according to the manufacturer's protocol. Briefly, RT reactions involve three main steps: primer annealing (65 °C, 5 min; 4 °C, > 1 min), DNA polymerization (23 °C, 10 min; 50–55 °C, 20 min) and enzyme deactivation (80 °C, 10 min). A mixture without RNA was used as a control for RT.

Real-time PCR (q-PCR) analysis

For real-time PCR analysis, 2 µL of cDNA samples (diluted 30-fold with Nuclease-Free Water), 0.6 µL of each 10 mM primer and 10 µL Power SYBRGreen Master Mix (Applied Biosystems) were amplified using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used were: ST6GALNAC1 “AGACCCCAGGAAGCTTTTCGAAGCACTCACCTGGTCACAG” (STAB VIDA), and β -

Actin “TACAGCTTCACCACCACAGCACTTTCATGGATGCCACAGG” (Sigma-Aldrich). The specificity of amplification was confirmed by melting curve analysis. A standard curve was determined for each gene using cDNA from HEK293 Δ COSMC + *ST6GALNAC1*, and results were presented as a ratio between the target gene and the housekeeping gene, β -Actin, and normalized to the positive control HEK HEK293 Δ COSMC + *ST6GALNAC1*.

→ **Supplementary Figure 1-4**

Supplementary Figure 1

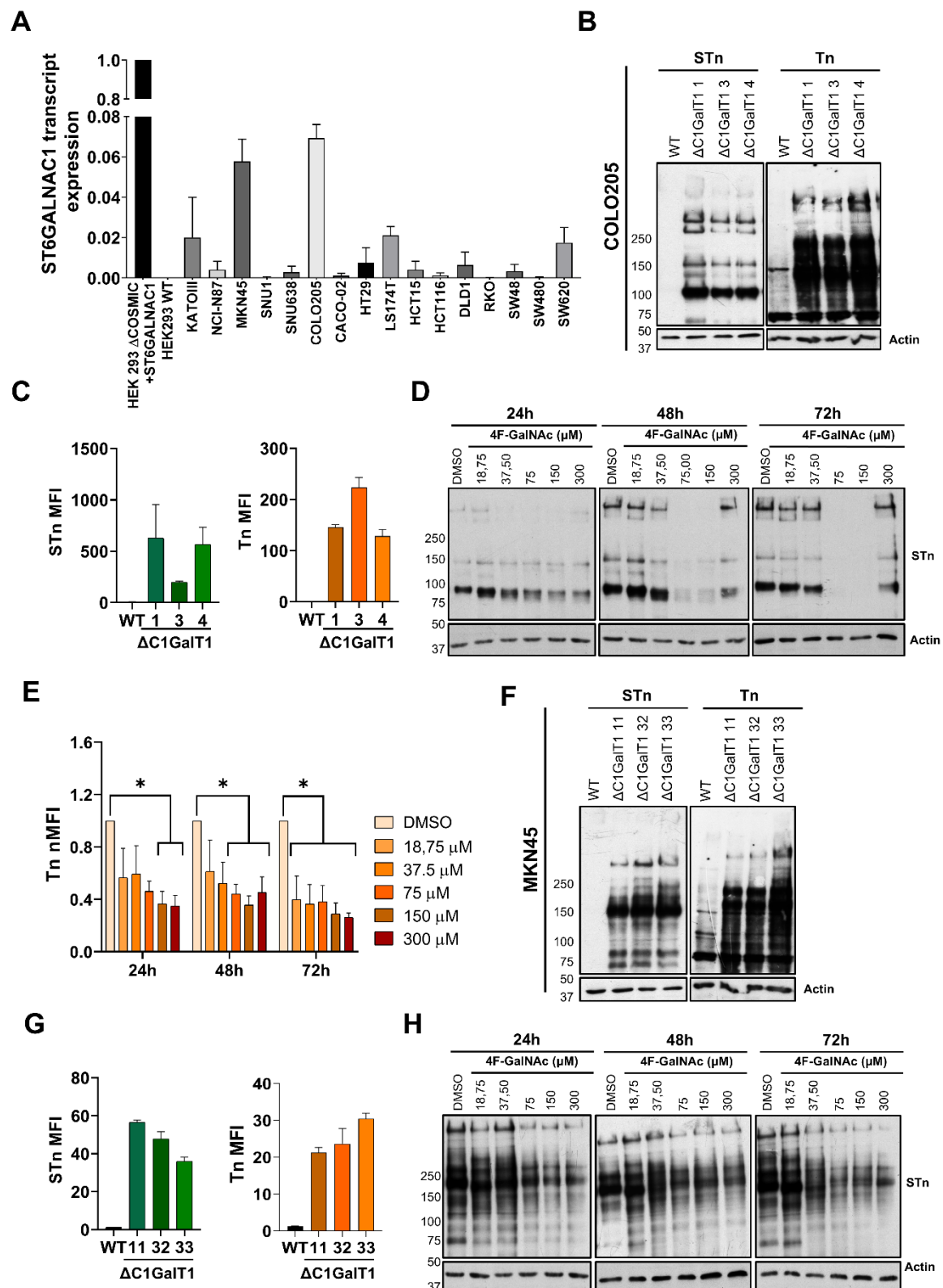
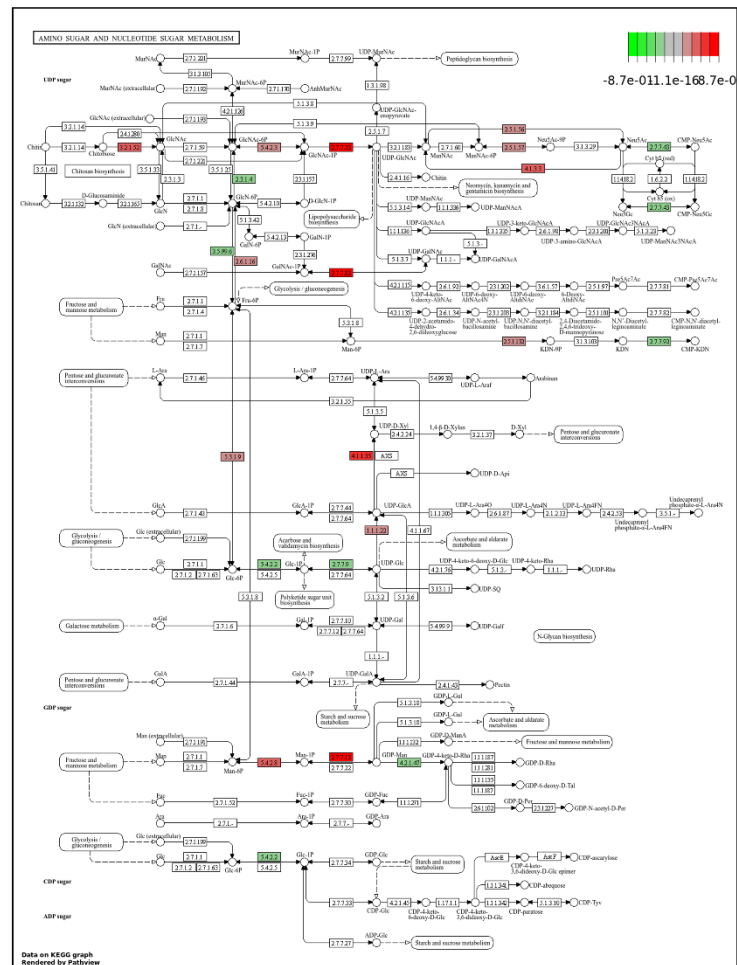


Figure S1: Characterization of Δ C1GalT1 GIC cell models and impact of 4F-GalNAc treatment in cancer-associated truncated O-glycan biosynthesis **A)** ST6GALNAC1 gene expression by real-time PCR (q-PCR) analysis, showing high expression in COLO205 and MKN45 cell lines. **B)** Western blot and **C)** Flow cytometry

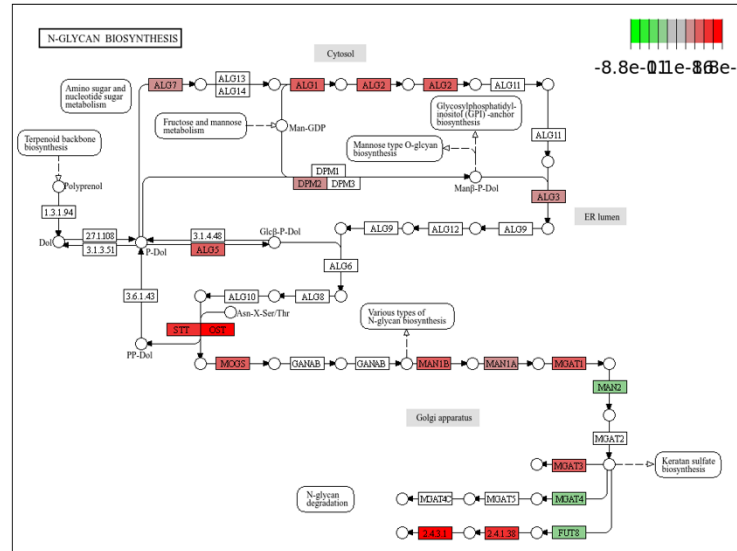
analysis of STn and Tn immunodetection in COLO25 WT and selected Δ C1GalT1 clones. **D)** Western blot analysis of STn in COLO205 Δ C1GalT1 cells after treatment with 4F-GalNAc in a concentration and time dependent manner, showing the reduction in STn detection after treatment. **E)** Flow cytometry analysis of Tn immunodetection after treatment with 4F-GalNAc in a concentration and time dependent manner, also showing reduction of Tn after treatment. **F)** Western blot and **G)** Flow cytometry analysis of STn and Tn antigen in WT and selected Δ C1GalT1 clones from MKN45 cell line. **H)** Western blot analysis of STn in MKN45 Δ C1GalT1 cells after treatment with 4F-GalNAc in a concentration and time dependent manner, showing a reduction in STn detection after treatment. Results are presented as mean $\pm$ SEM of three independent experiments. *p<0.01 4F-GalNAc-treated vs DMSO-treated group. MFI: Median fluorescent intensity. nMFI: Median fluorescent intensity normalized to DMSO

A

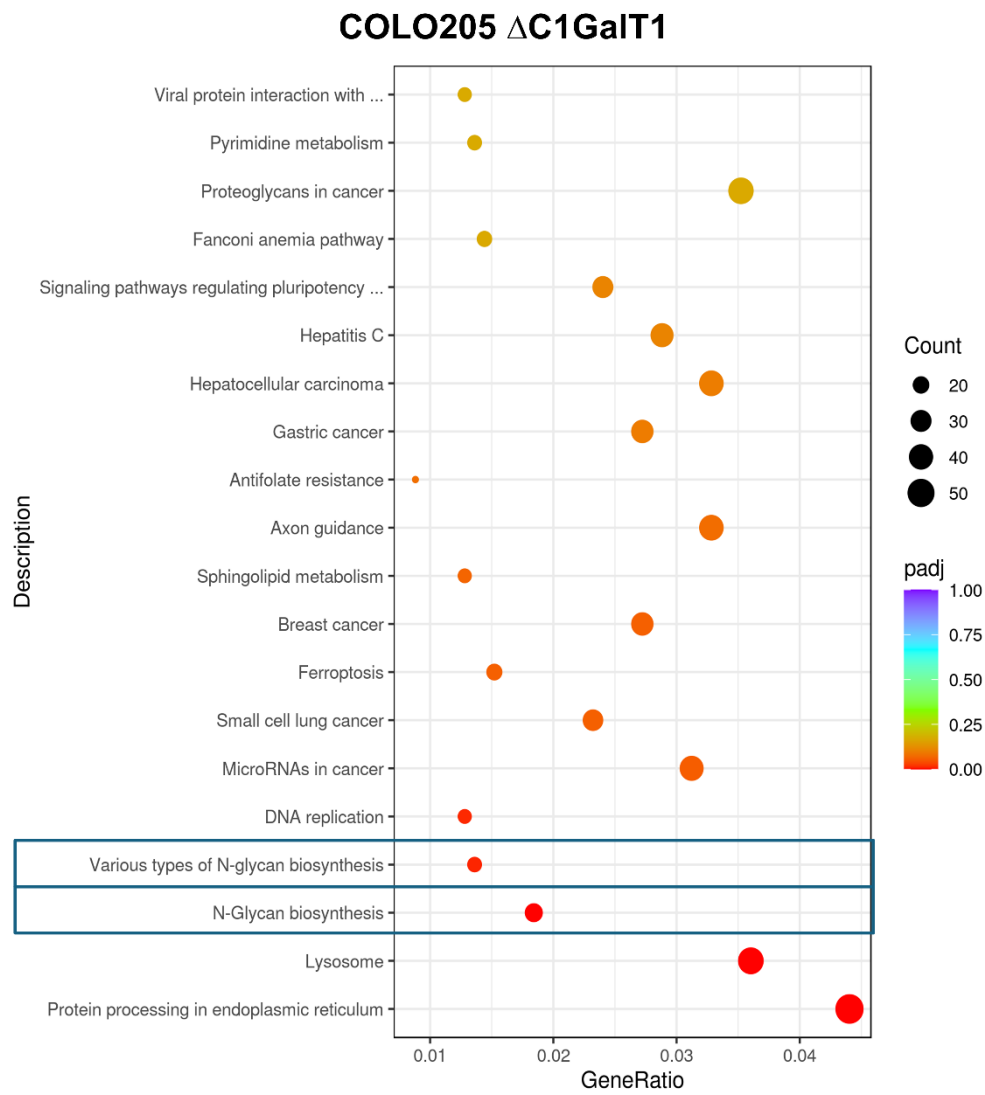
COLO205 WT



B

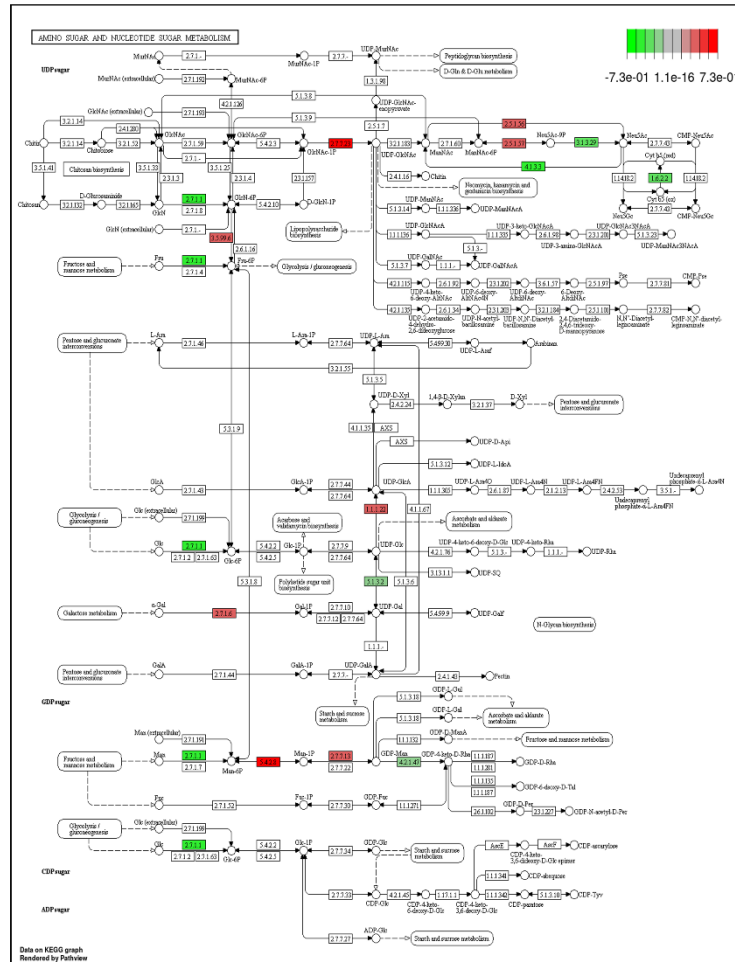


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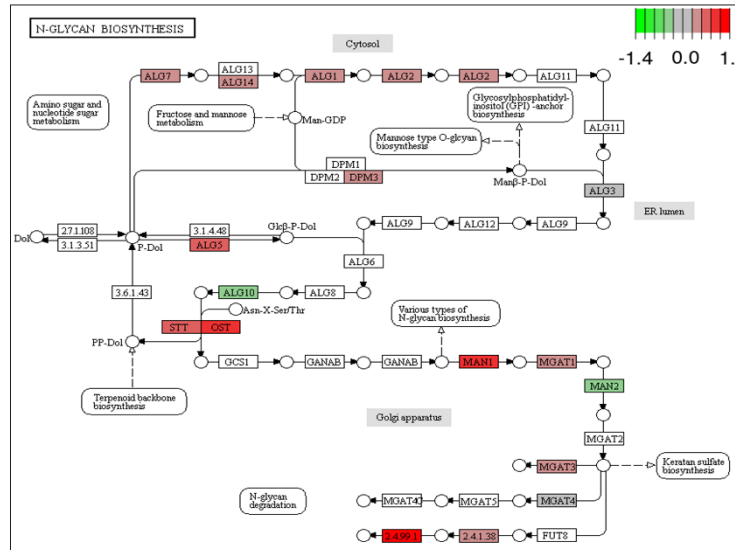


D

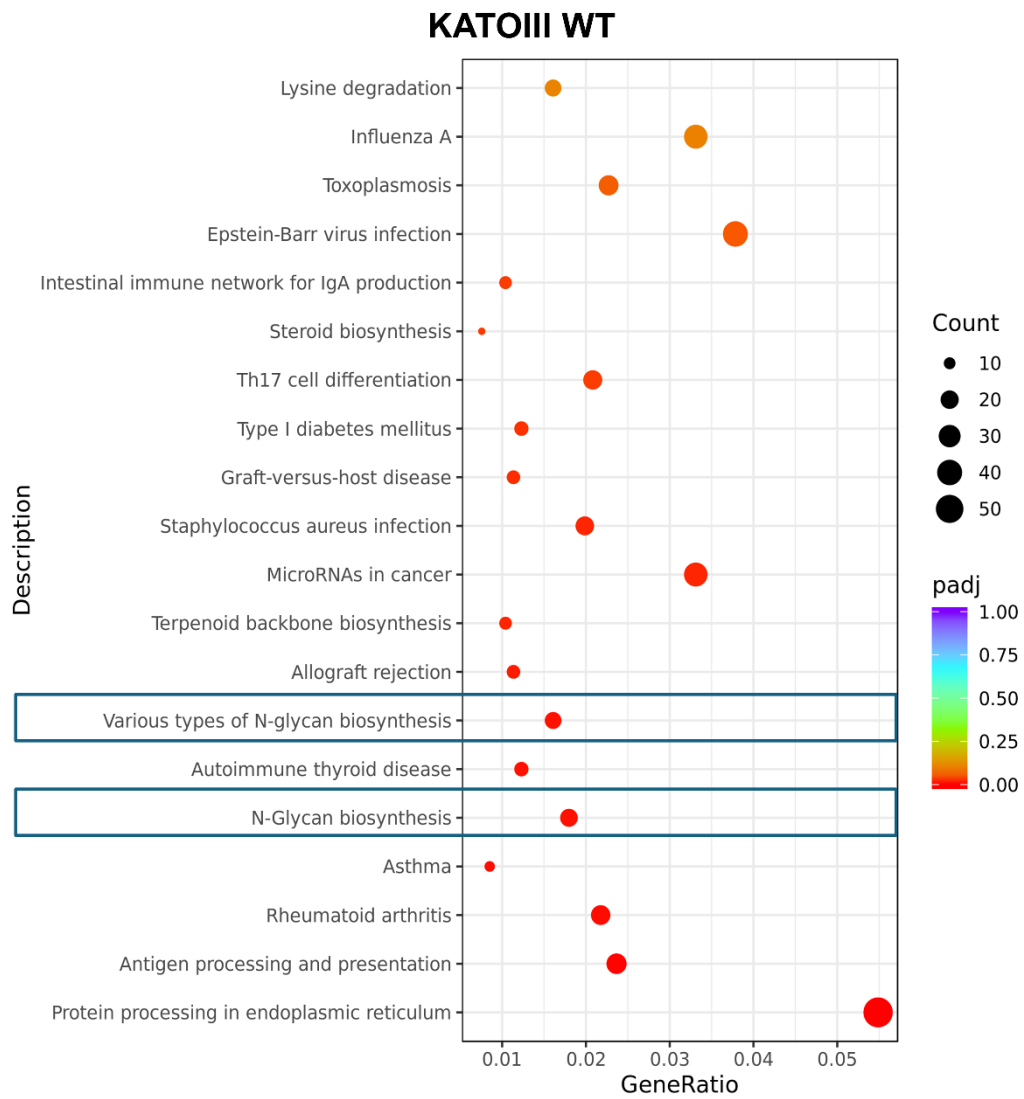
COLO205 Δ C1GalT1



E

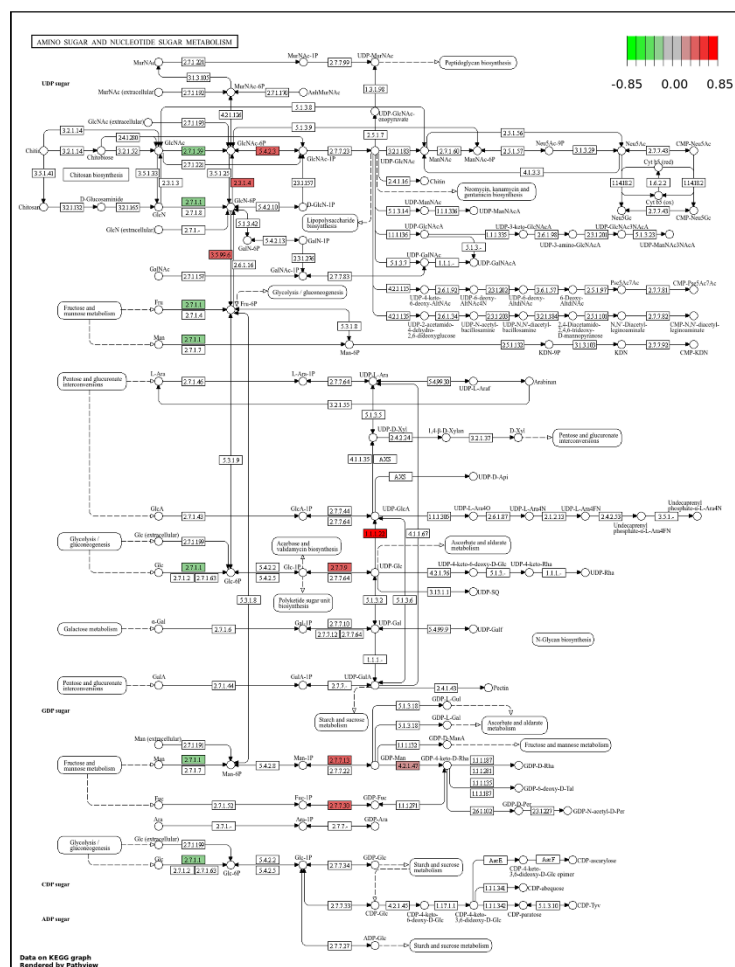


F



G

KATOIII WT



H

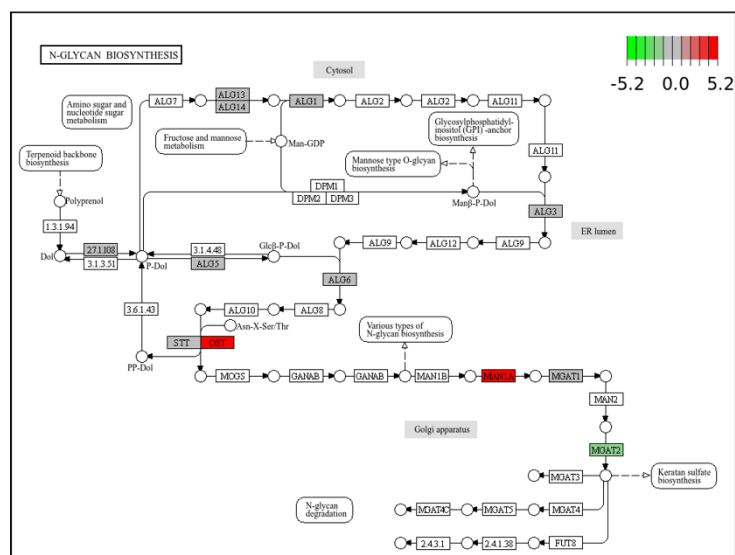


Figure S2: KEGG analysis of RNA sequencing comparing 4F-GalNAc and DMSO-treated COLO205 WT, COLO205 Δ C1GalT1 and KATOIII WT cells. A) Amino sugar and nucleotide sugar metabolism pathway, showing alterations in several genes, especially associated with GalNAc, GlcNAc and Neu5Ac sugar donors in COLO205 WT cells treated with 4F-GalNAc. B) N-glycan biosynthesis pathway presenting several upregulated genes in COLO205 WT cells treated with 4F-GalNAc C) General analysis showing gene alteration in the

biosynthesis of *N*-glycans on COLO205 Δ C1GalT1 cells treated with 4F-GalNAc. **D)** Amino sugar and nucleotide sugar metabolism pathway of 4F-GalNAc vs DMSO treated COLO205 Δ C1GalT1 cells, showing the alteration of several genes, especially associated with GalNAc, GlcNAc, and Neu5Ac sugar donors. **E)** *N*-glycan biosynthesis pathway presenting several upregulated genes in COLO205 Δ C1GalT1 treated cells **F)** General analysis showing gene alteration on the biosynthesis of *N*-glycans in KATOIII cells treated with 4F-GalNAc. **G)** Amino sugar and nucleotide sugar metabolism pathway of KATOIII 4F-GalNAc vs DMSO treated cells, showing the alteration of several genes, especially associated with GalNAc and GlcNAc sugar donors. **H)** *N*-Glycan biosynthesis pathways showing some gene alterations. Red corresponds to upregulated genes and green corresponds to downregulated genes.

Supplementary Figure 3

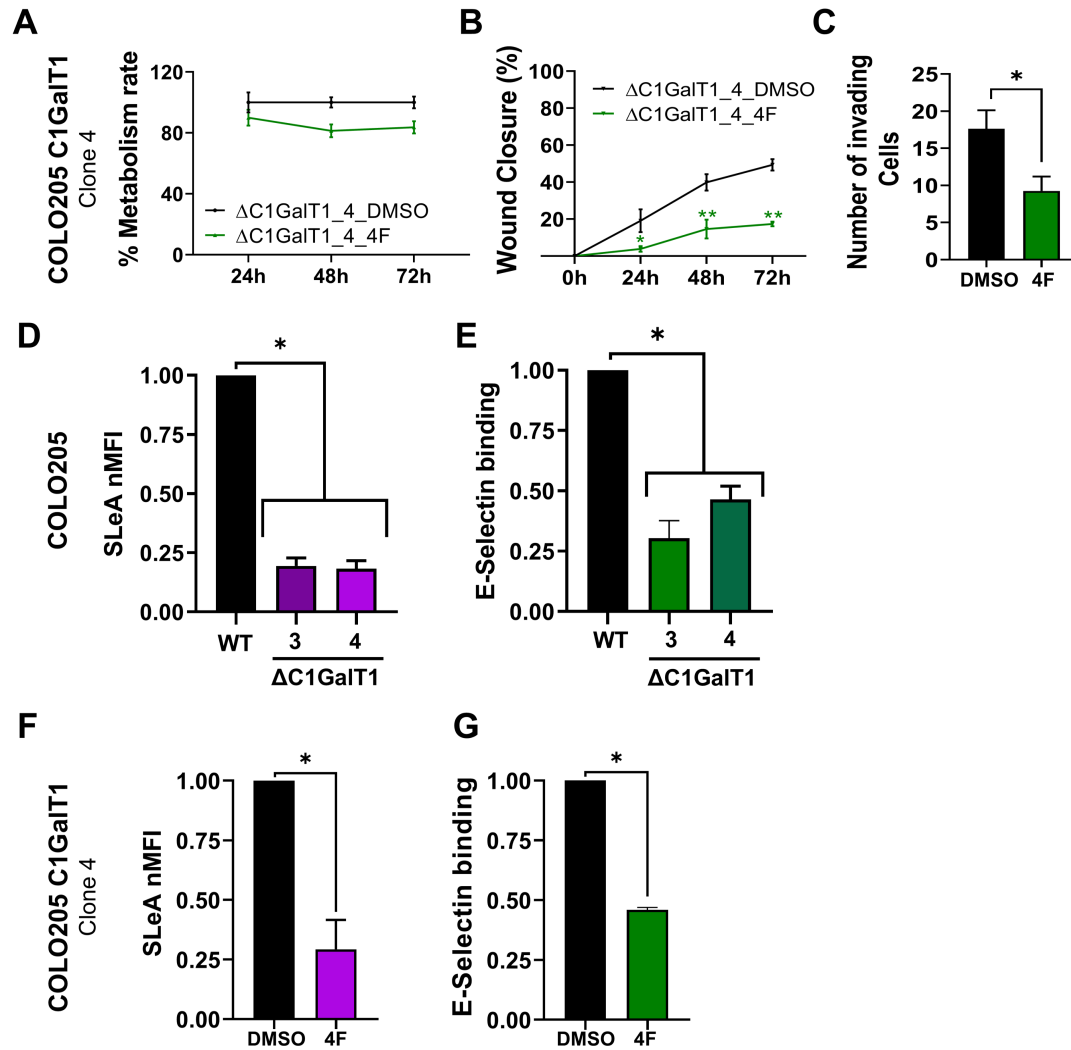


Figure S3: Biological impact of 4F-GalNAc in COLO205ΔC1GalT1 cells. **A)** Cell metabolism (evaluated by MTT assay) in COLO205ΔC1GalT1 clone 4 treated with 4F-GalNAc during 72h, showing a reduction in the metabolic activity of treated cells. **B)** Wound healing assay in COLO205ΔC1GalT1 clone 4 during 72h showing the reduction in cell motility after treatment with 4F-GalNAc. **C)** Invasive capacity of COLO205ΔC1GalT1 clone 4 treated with 4FGalNaAc during 48h showing a reduction in the invasive capacity of treated cells. **D)** Flow cytometry analysis of SLeA in COLO205 ΔC1GalT1 clones 3 and 4 showing a reduction in SLeA immunodetection after C1GalT1 KO. **E)** E-selectin binding in COLO205 ΔC1GalT1 clones 3 and 4 showing a reduction in binding in KO cells. **F)** Flow cytometry analysis of SLeA expression and **G)** E-Selectin binding in COLO205ΔC1GalT1 clone 4 cells after treatment with 4F-GalNAc showing a reduction in SLeA expression and E-Selectin binding after treatment. Results are presented as mean ± SEM of three independent experiments. *p<0.01 4F-GalNAc-treated vs DMSO-treated group. nMFI: Median fluorescent intensity normalized to DMSO.

Supplementary Figure 4

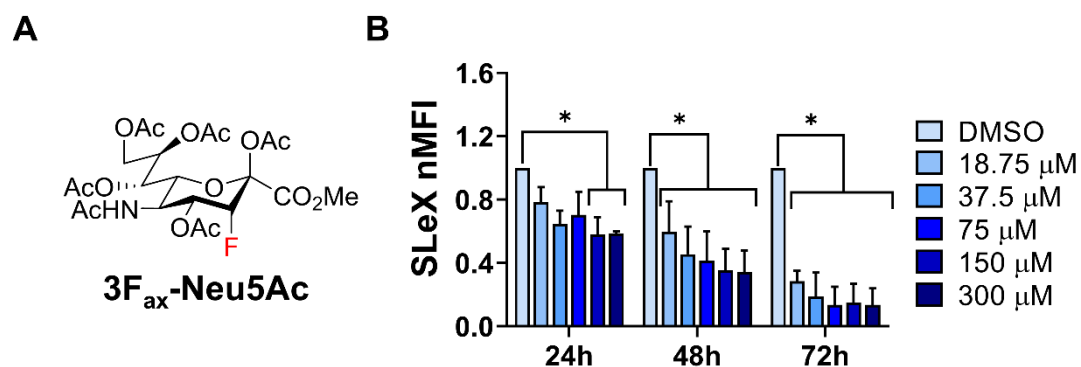


Figure S4: Impact of 3F_{ax}-Neu5Ac in SLeX expression in COLO205 WT cells. **A)** Chemical structure of 3F_{ax}-Neu5Ac. **B)** Flow cytometry analysis of SLeX immunodetection in COLO205 WT after treatment with 3F_{ax}-Neu5Ac showing a reduction in all tested concentrations over time. Results are presented as mean $\pm$ SEM of three independent experiments. * $p < 0.01$ 3F_{ax}-Neu5Ac-treated vs. the DMSO-treated group. nMFI: Median fluorescent intensity normalized to DMSO.

Chapter 5

General Discussion

Final Remarks and Future Perspectives

General Discussion

With an estimated 20 million new cases and nearly 10 million deaths annually, cancer is a major global health burden [1]. According to WHO, the incidence of new cancer cases is projected to increase by 77% in 2050. This is driven primarily by an aged population and increased exposure to risk factors, many of which are linked to socioeconomic development. Additionally, cancer-related deaths are expected to rise by 93%, particularly among men, with the greatest impact in low-income countries. Particularly, new cancer incidence is also becoming more common in younger individuals [2].

GIC, especially GC and CRC, are among the top five most diagnosed and deadly cancers globally [1]. Diagnosing these cancers is particularly challenging, as symptoms often appear only in the later stages of the disease [3,4]. Moreover, there is a lack of non-invasive protocols for early diagnosis of the disease [5,6]. Currently, only more invasive exams such as endoscopic examination for GC or colonoscopy for CRC remain reliable diagnostic tools [7,8]. Despite substantial investments in early diagnosis programs, further efforts are required, particularly in low-income countries, as well as in developing less invasive, but effective, diagnostic methods.

When cancer is diagnosed at an early stage, surgery offers the best chance for a cure. However, when diagnosis occurs in the latter stages of the disease, and surgery is no longer possible, the primary treatment options for most patients consist of chemotherapy and radiotherapy. Some patients may also be eligible for treatment with mAbs such as anti-HER2 or anti-PD-L1 for GC, and anti-EGFR for CRC [7,9-11]. Nevertheless, this approach is restricted to a small subset of cancer patients, mainly at advanced stages, and has associated toxic side effects and therapy resistance [12,13]. One key factor contributing to therapy resistance is glycosylation, as it can hinder the binding of the mAb to the target protein [14]. Furthermore, glycosylation is also associated with resistance to chemotherapeutic drugs, such as 5-FU [15]. In addition to the contribution of glycosylation to therapies resistance, altered glycan biosynthesis is a well-established feature in cancer, with the detection of these cancer-associated glycans used currently as serological biomarkers.

Glycosylation, the enzymatic biosynthesis of carbohydrates/glycans that can be found freely or attached to proteins, lipids or RNA, is mediated by the organized action of a collection of different GTs and glycosidase enzymes. This leads to a myriad of complex structures that are essential for normal cellular function, as they are involved in several biological processes. Consequently, it is not surprising that alterations in the glycosylation process can be found in pre-cancerous and cancerous cells, which results in the overexpression of several glycostructures and/ or *de novo* expression of glycans not

typically found in healthy conditions [16]. As a result, targeting glycosylation pathways holds promise for enhancing the effectiveness of cancer treatments, reducing resistance, and developing novel targeted therapies. Indeed, several clinically used biomarkers are highly glycosylated proteins such as CEA or CA125 (MUC16), or glycan structures such as CA19-9 (SLeA) or CA72-4 (STn), underscoring the importance of glycosylation in cancer [17].

In cancer cells, one of the most commonly found alterations is the overexpression of STs, which are responsible for the biosynthesis of sialylated Lewis antigens such as SLeX or SLeA [16]. Focusing on SLeX, this terminal tetrasaccharide structure can be found in leukocytes, where it binds to selectins during an inflammatory response. Cancer cells exploit this mechanism, overexpressing SLeX to facilitate cancer cell binding to endothelial cells, rolling in the endothelium, and extravasate from blood vessels to a metastatic site [18,19]. Consequently, SLeX is found in several types of cancer, including GC and CRC, and has a high association with increased aggressiveness of cancer cells, increased metastasis formation, and a poor prognosis of patients [20-24]. Inhibiting SLeX biosynthesis has shown promising results in limiting cancer progression and metastatic behavior [22,23]. As a result, SLeX represents a promise as a therapeutic target, and developing strategies to inhibit its biosynthesis offers significant potential as a novel therapeutic approach.

Accordingly, in this thesis that focuses on SLeX as a therapeutic target, we propose two primary objectives: 1) Elucidate the main ST involved in the biosynthesis of SLeX in GIC cells as explored in **Chapter 2**. This is essential to deeper understand the GTs involved in the synthesis of aberrant glycans and can help improve the design of new therapies. 2) Discover novel therapeutic molecules that target cancer-associated glycan biosynthesis, mainly SLeX, in GIC cells. Two distinct strategies were employed, leading to the identification of two promising drug candidates with different mechanisms of action, detailed in **Chapters 3** and **Chapter 4**.

Starting with the first goal, the terminal addition of sialic acid in SLeX glycans is carried by members of the ST3Gal family, specifically by ST3GalIII, ST3GalIV, and ST3GalVI. Previous studies from our group, using a cell model overexpressing ST3GalIV, show the role of this enzyme in SLeX expression in MKN45 GC cells [25] and its association with the activation of the c-MET signaling pathway [20]. It is also known that ST3GalIII preferentially acts on type I glycan chains giving rise to the SLeX isomer SLeA. Nevertheless, some studies have demonstrated the role of ST3GalIII in SLeX expression, for instance in cancer [23,26]. Therefore, we wanted to unveil the role of ST3GalIV in SLeX expression and the malignant properties of GI cancer cells. For that, in **Chapter 2**, we established Knock-Out (KO) GIC cell models to understand the specific contribution of distinct ST3Gal enzymes. From a panel of thirteen GIC cell lines, we selected five cell lines that were positive for SLeX: KATOIII and NCI-N87 (GC) and HT-29, LS174T and COLO205

(CRC). Notably, the SLeX-positive cell lines presented higher expression levels of the ST3Gal4 gene in tested cell lines. The CRC DLD1 cell line was an exception as it presented high levels of ST3GalIV and was negative for SLeX. We started our study by KO ST3GAL4 gene in the selected cell lines, by CRISPR-Cas9, and observed a complete abrogation of SLeX expression in four of the selected cell lines, being LS174T the only exception. The SLeX abrogation by ST3GAL4 gene KO also led to a significant reduction of the migratory capacity in KO cells, corroborating our previous findings on the SLeX expression induced by ST3GalIV alters the biological behavior of cancer cells [20,23]. Interestingly, the ST3Gal4 KO also reduced SLeA levels and increased expression of the non-sialylated Lewis X and A structures, suggesting that ST3GalIV may contribute to the sialylation of both type I and type II Lewis glycan chains. Although the literature supports that ST3GalIII is the major enzyme in SLeA biosynthesis, our study and others show that, in some cases, a compensatory coordinated action of ST enzymes could be responsible for the synthesis of this antigen. Similarly, this can occur with the synthesis of SLeX. Although the KO of the ST3GalIV enzyme was effective in abolishing SLeX in most positive cell lines, we found an exception with the LS174T cell line. In these cells, there is a likely glycolipid-associated SLeX expression, since SLeX was completely reduced at the protein level, by western blot, but not by immunofluorescence. Indeed, the SLeX/A found in this cell line was described to be mainly in gangliosides [27]. Given that evidence, and since many studies associate ST3GalVI as the enzyme mainly involved in the sialylation of type II glycan chains mainly on glycolipids, we performed a KO of the ST3GAL6 gene. Indeed, ST3Gal6 presented high levels in LS174T in our analysis, corroborating this hypothesis. Our results showed that only the double KO of ST3GalIV/VI was able to fully abrogate SLeX detection and impair the motility capacity of LS174T cells. Interestingly, although both single KO could not abolish completely SLeX expression and consequently impact the migration of cells, it could inhibit SLeX detection at protein levels, which made us speculate about a possible enzymatic interaction or some type of dependency mechanism between both enzymes, essential for the synthesis of SLeX in proteins in the LS174T cells. It would be interesting to explore the interaction between both enzymes and if similar cases can be found in cells from other cancer types. Nevertheless, at the end of this chapter, we demonstrate the main role of ST3GalIV in the biosynthesis of SLeX in GIC cells, and its association with cellular motility. Additionally, we showed that, in particular cases, this enzyme is not the only responsible for the addition of α 2,3 sialylation in SLeX biosynthesis.

Having cleared the role of ST3GalIV in SLeX expression, we proceed to the second goal of this thesis: identify novel therapeutic molecules that target cancer-associated glycans. Using SLeX as a target, we employ two vastly different strategies, explored in **Chapter 3** and **Chapter 4**.

In **Chapter 3**, we establish a fluorescent HTS method to identify compounds that inhibit the synthesis of SLeX expression in cancer cells. HTS is widely used in drug discovery as it simultaneously permits the screening of thousands of compounds at greater efficiency and lower costs. Using glycosylation as targets in HTS is still poorly explored. However, several interesting approaches have been employed for glyco-specific HTS assays, especially after the expanded use of fluorescence methods [28]. In our HTS, we used COLO205 cells, which are 100% positive for SLeX detection, and analyzed the fluorescent intensity of each well in the presence of tested compounds. We screened 7836 compounds, comprising two distinct libraries, and identified monensin as the most promising hit. Monensin is a polyether ionophore, largely used in veterinary practice. This was not the first time monensin arose from an HTS, principally in demonstrating its potential as a novel therapy for several types of cancer [29-41]. This is the first time, as far as we know, that monensin was identified in HTS that used glycans as a target. We showed that monensin inhibits SLeX expression in the CRC cell line COLO205, and in the GC cell line KATOIII. Mechanistically, as an ionophore, monensin deregulated cation influxes, specifically Na^+ and Ca^{2+} , disrupting the Golgi compartment, compromising its normal function and, consequently, impacting protein glycosylation, processing, and secretion. This was supported by an increased expression of the truncated O-glycan Tn antigen and an impairment of SLeX carriers at the cell membrane and the secreted forms, and an abnormal expression and mislocalization of E-cadherin in treated cells. Biologically, tested monensin concentrations selectively altered the metabolism of SLeX-positive cells, with no significative alterations observed in four tested SLeX-negative GIC cells. We hypothesize that the anionic charge conferred by sialic acids at the cell surface of SLeX-positive cancer cells [42] potentially facilitates monensin's uptake and intracellular transport, making these cells more sensitive to this compound. Moreover, monensin reduced cell motility and invasive capacity of SLeX-positive GIC cells and reduced tumor invasion and growth across various *in vivo* models. Monensin was also validated in a pre-clinical model of GC patient-derived organoids (PDOs) and in the patient-derived organoids xenograft (PDOXs) mouse model. Moreover, combination with 5-FU, a standard chemotherapeutic drug used in GIC treatment, showed a synergistic action of both drugs, leading to an improved effect in inhibiting tumor growth without toxicity effects into mice.

The main issues with several drugs that arise from HTS are toxicity and lack of specificity. In our case, SLeX-positive cells are more sensitive to monensin, as it can negatively impact cellular metabolism, which was not observed in SLeX-negative cells. In PDOs, we also found a positive correlation of monensin IC₅₀ and high levels of SLeX expression. Furthermore, we did not observe any sign of stress or toxicity in mice, even with long periods of monensin administration. Several studies proposed monensin as a

repurposed drug for cancer therapy. However, studies addressing the implementation of monensin as new drug in humans, such as tolerable doses, administration routes, among others, are inexistent, which explains why monensin never reached a clinical trial. Moreover, reports claiming toxicity after monensin intake in humans raise worries, however, these consist of uncontrolled dosages of monensin veterinary formulation [37,43-46]. Therefore, it is important to establish safe and controlled monensin dosage, formulation, and administration protocols to be considered for its clinical use.

Overall, our findings highlight monensin's potential as a cancer therapy, specifically for targeting SLeX-positive GIC. We demonstrated monensin ability to inhibit SLeX expression and its associated malignant properties, reducing tumor invasion and growth. We also disclose a possible mechanism of action, and our pre-clinical results highlight its potential as a therapeutic candidate for cancer treatment.

In **Chapter 4**, we evaluate the impact of different fluorinated GalNAc (3F-, 4F- and 6F-GalNAc) on the biosynthesis of cancer-associated O-glycans, such as SLeX, Tn and STn. Approaches using chemically modified sugar analogs have been suggested as a strategy to inhibit glycosylation pathways [47]. In addition, the use of fluorinated drugs has emerged as one of the fastest-growing classes of organic compounds in the last decades [48]. Therefore, several F-MAs, have shown promising anti-cancer effects both *in vitro* and *in vivo* [49-52]. However, HexNAc analogs, particularly fluorinated GalNAc are relatively unexplored. Hence, in this chapter, we pointed out the capacity of 4F-GalNAc in reducing the immunodetection of cancer-associated O-linked glycans such as Tn, STn and SLeX. Using three SLeX-positive GIC cell lines that present SLeX at distinct glycoconjugates, we demonstrated the capacity of 4F-GalNAc to specifically reduce the immunodetection of O-linked SLeX. Moreover, using C1GalT1 KO cell models, where cells have an increased expression of easily detectable Tn and STn O-glycan antigens, 4F-GalNAc also led to a significant reduction in their immunodetection. Moreover, both 3F- and 6F-GalNAc did not show consistent inhibition of SLeX, Tn or STn in our cell models, despite the latest being described as effective in reducing several glycan structures [53].

Our findings suggest that 4F-GalNAc inhibits glycosylation by impairing the *de novo* synthesis of sugar donors, as demonstrated by changes in genes associated to the metabolism of GalNAc, GlcNAc, sialic acids, and Man. This observation aligns with other studies, which showed that F-MAs inhibit the production of endogenous UDP-GalNAc/GlcNAc, CMP-Neu5Ac, and GDP-Man [54,55]. Also, authors showed that 4F-GalNAc can be incorporated into glycan structures on multiple cellular glycoproteins [56]. However, despite a small degree of incorporation described for some non-fluorinated HexNAc analogs [57,58], in the case of 4F-GlcNAc and 6F-GalNAc no incorporation was observed [53,59]. Preliminary mass spectrometry structural analysis (data not shown)

pointed to no incorporation of 4F-GalNAc in glycan structures in our models. In addition, we have observed a reduction of Tn antigen detection, similar to STn, which made us believe the incorporation of 4F-GalNAc is not the mechanism behind 4F-GalNAc inhibitory effects. Nevertheless, confirmation studies such as the evaluation of the natural sugar donor levels after treatment, and enzymatic assays using recombinant ppGalNAcTs are required to fully confirm the mechanism of action of 4F-GalNAc. Additionally, 4F-GalNAc treatment showed the upregulation of several genes involved in the *N*-glycosylation biosynthesis pathway. Previous studies also showed *N*-glycan alterations after 4F-GalNAc treatment [53,55,56]. In our study, the observed alterations of the sugar donor level such as UDP-GlcNAc and GDP-Man may be the main cause of the effects on *N*-glycan biosynthesis. However, deep characterization of *N*-glycan structures in cells treated with 4F-GalNAc is mandatory to better define the impact of this F-MA in the general glycosylation of the cells.

Furthermore, 4F-GalNAc had a significant impact on the biological behavior of cancer cells, especially affecting important features associated with the metastasis process. For instance, the reduction of SLeX and SLeA epitopes with 4F-GalNAc treatment largely affected E-Selectin binding. Also, a reduction in the migration and invasion capacity of cells was observed. This could be due to the observed alterations in RTK activation. Indeed, these receptors are a subclass of tyrosine kinases that control a wide range of complex biological functions, including cell growth, motility, differentiation, and metabolism [60]. Furthermore, RTKs are transmembrane glycoproteins and, consequently, alteration in their normal glycosylation pattern impacts their activation and the subsequent signaling pathways [61,62]. Indeed, the effect of 4F-GalNAc on cancer cells' glycosylation remodeling led to alterations of specific RTKs activation. Several studies describe similar alterations in the activation of identified RTKs after glycosylation pattern modification [20,63-69]. Consequently, this also contributes to the biological impact observed in treated cells. Nevertheless, validation is needed to fully understand the alterations throughout the several signaling pathways affected, and what specific changes in glycosylation were the main responsible for these alterations.

Using a xenograft mouse model, we showed the *in vivo* capacity of 4F-GalNAc in impairing tumor growth. As a control, we used 3F_{ax}-Neu5Ac and similar effects were observed using both analogs, with a tendency for 4F-GalNAc to be more efficient. The main concern in using F-MAs is related to toxic effects. 3F_{ax}-Neu5Ac is known to cause renal failure in mice [70], and *in vivo* testing is normally done by intratumoral administration [50,71]. Nevertheless, the promise of F-MAs as therapeutic agents has already been demonstrated for instance using 2F-Fuc. An oral administration of 2F-Fuc entered a Phase I clinical trial for patients with advanced solid tumors (NCT 02952989) and got great results in suppressing tumor growth. However, due to associated thromboembolic events, the study

had to be terminated [72]. Regarding 4F-GalNAc, intravenously injection of higher doses did not cause toxicity to mice, although there was a small-time window of testing [56]. Furthermore, we did not observe any toxicity or signs of distress in mice in our experiments. Nevertheless, specific studies are needed to clarify the pharmacokinetics and toxicity of 4F-GalNAc and even employ further modifications to improve potency and specificity. Indeed, modifications could be employed to 4F-GalNAc. For instance, addition of 1-O-deacetylation to 4F-GalNAc structure showed an increased efficacy in inhibiting the proliferation of human prostate cancer cells [73]. The chemical addition of an organometallic moiety at the C2 position of 4F-GalNAc also showed increased cytotoxicity against cisplatin-resistant breast and ovarian cancer cells [74]. Moreover, other modifications described in other F-MAs could also be applied to 4F-GalNAc. The addition of a carbamate residue into the C5 position of 3F_{ax}-Neu5Ac was demonstrated to achieve higher inhibitory potency. A further introduction of a photolabile ortho-nitrobenzyl group, for inducible and local inhibition of sialoglycans biosynthesis, is an example of how to improve the specificity of a F-MA [75]. Another example is the addition of a phosphate group at the C1 position that showed an increased potency and prolonged inhibitory effect of 2F-Fuc [51,76].

Hence, in this study, we demonstrated the capacity of 4F-GalNAc to inhibit cancer-associated O-glycans biosynthesis, which affected associated malignant biological behaviors *in vitro* and *in vivo*. This opens an opportunity for the development and implementation of a new and effective cancer therapy.

In this thesis, employing **two different strategies**, we identify and propose two different drugs that target aberrant glycosylation and could be applied as potential cancer therapy: **monensin** and **4F-GalNAc**. Both compounds successfully reduce SLeX expression and its associated biological malignancy using GIC models. Although we suggest their use for the treatment of GIC, aberrant glycosylation is linked to malignancy in various tumor types, suggesting broader therapeutic applicability. Several studies tested monensin with beneficial effects to distinct tumors. Similarly, 4F-GalNAc and other F-MAs were also validated as glycan inhibitors in various cancer cell types.

Nonetheless, as with most proposed cancer drugs, one of the main challenges is achieving cancer-cell specificity to minimize toxicity. As previously mentioned, human intake of uncontrolled doses of monensin resulted in high toxicity [37,43-46]. Moreover, although not reported for 4F-GalNAc, some F-MAs showed toxic effects in *in vivo* models [70] and side effects in humans [72]. Taking this into consideration, recent reports already account for this specific cell-targeting obstacle. A targeted delivery system where 3F_{ax}-Neu5Ac was encapsulated in nanoparticles was already established and showed good efficacy in reaching cancer cells and in preventing metastasis outgrowth, with neglected effects on normal cells using mouse model [77]. Therefore, this system could be easily

adapted with other F-MAs to tackle different tumors. Monensin was also encapsulated into PLGA nanoparticles to be more effective in inhibiting the growth of *P. falciparum* [78]. Moreover, encapsulation of monensin together with immunotoxins (ITs) potentiated the action of ITs by 40 to 50 times against CRC cell lines [79,80].

In addition to the nanoparticles approach, we envision that the establishment of antibody-drug conjugates (ADCs) coupling monensin or 4F-GalNAc to mAbs that specifically recognize cancer-specific antigens can be a clever strategy to avoid side effects. ADCs can minimize toxicity by selectively delivering the payload to the tumor site. Bertozzi's lab initially showed the promise of ADCs in targeting glycans in cancer. Sialidases, when linked to antibodies that specifically recognize Her2 on cancer cells, strip the sialic acids from the cancer cell's glycocalyx, triggering an immune response that promotes cell destruction, representing a promising approach to cancer immune therapy *in vivo* [81]. Furthermore, an anti-My9 antibody-conjugated monensin liposome was described to potentiate cytotoxicity in leukemia cells [82]. Despite challenges, such as choosing suitable linkers and payloads, several ADCs are making significant progress [83,84]. These advancements demonstrate the potential of ADCs in cancer therapy despite the complexities involved.

Final Remarks and Future Perspectives

Glycosylation in healthy cells is a highly controlled process, and alterations in this process are often linked to cancer. In cancer cells, the expression of several glycan alterations is well-documented. For instance, SLeX, a sialylated Lewis antigen commonly found overexpressed in cancer, has a crucial role in cancer progression and aggressiveness, being associated with metastasis formation and a worse patient prognosis. Reducing or abolishing SLeX biosynthesis has shown promising results in limiting metastatic behavior. Therefore, developing strategies that inhibit the biosynthesis of SLeX offers a promising therapeutic approach. Furthermore, a deeper understanding of the specific GTs involved in the synthesis of this aberrant glycan could lead to the development of more selective and effective cancer therapies.

The results obtained in this thesis allow the depiction of the following conclusions:

- 1) ST3GalIV is the main ST involved in the biosynthesis of SLeX in GIC cells and, consequently, playing a key role in cellular motility. However, in certain cases, ST3GalIV is not the sole enzyme responsible for SLeX biosynthesis.
- 2) Employing two different strategies, we identify and propose monensin and 4F-GalNAc as novel drugs to target aberrant glycosylation in cancer.
- 3) Monensin disrupts Golgi function, which impacts SLeX expression and its cancer-associated malignant properties. Its potential as an anti-cancer drug was demonstrated by *in vivo* xenografted models and in the pre-clinical model of GC patients-derived organoids.
- 4) 4F-GalNAc interferes with the biosynthesis of cancer-associated O-glycans, impacts RTKs activation, and reduces aggressive cancer cell biological behavior. Its efficacy in reducing tumor growth, using an *in vivo* model, supports its potential as a novel approach for developing a new cancer therapy,

However, regarding the proposed drugs, several questions remain unresolved. For instance, despite the promising results, there are no studies that establish monensin safe concentrations for human use. Therefore, further testing is needed to bring monensin closer to clinical trials. Additionally, the 4F-GalNAc mechanism of action and its impact on *N*-glycosylation still need to be clarified. Comprehensive pharmacokinetic and toxicity profiles for 4F-GalNAc are also needed.

Finally, the development of a targeted delivery system, such as an ADC, could address the main challenge of the lack of specificity towards cancer cells. Such delivery systems could significantly reduce potential side effects, improving the safety and efficacy of the treatment.

References

- [1] Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2024 May-Jun;74(3):229-263.
- [2] Koh B, Tan DJH, Ng CH, et al. Patterns in Cancer Incidence Among People Younger Than 50 Years in the US, 2010 to 2019. *JAMA Netw Open*. 2023 Aug 1;6(8):e2328171.
- [3] Sexton RE, Al Hallak MN, Diab M, et al. Gastric cancer: a comprehensive review of current and future treatment strategies. *Cancer Metastasis Rev*. 2020 Dec;39(4):1179-1203.
- [4] Jelski W, Mroczko B. Biochemical Markers of Colorectal Cancer - Present and Future. *Cancer Manag Res*. 2020 Jun;12:4789-4797.
- [5] Kuipers EJ, Grady WM, Lieberman D, et al. Colorectal cancer. *Nat Rev Dis Primers*. 2015 Nov 5;1:15065.
- [6] Matsuoka T, Yashiro M. Biomarkers of gastric cancer: Current topics and future perspective. *World J Gastroenterol*. 2018 Jul 14;24(26):2818-2832.
- [7] Smyth EC, Nilsson M, Grabsch HI, et al. Gastric cancer. *Lancet*. 2020 Aug 29;396(10251):635-648.
- [8] Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol*. 2019 Dec;16(12):713-732.
- [9] Lordick F, Carneiro F, Cascinu S, et al. Gastric cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2022 Oct;33(10):1005-1020.
- [10] Argiles G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2020 Oct;31(10):1291-1305.
- [11] Cervantes A, Adam R, Rosello S, et al. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2023 Jan;34(1):10-32.
- [12] Zhang J, Qiu W, Zhang W, et al. Tracking of trastuzumab resistance in patients with HER2-positive metastatic gastric cancer by CTC liquid biopsy. *Am J Cancer Res*. 2023 Nov;13(11):5684-5697.
- [13] Xie YH, Chen YX, Fang JY. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct Target Ther*. 2020 Mar 20;5(1):22.

- [14] Duarte HO, Rodrigues JG, Gomes C, et al. ST6Gal1 targets the ectodomain of ErbB2 in a site-specific manner and regulates gastric cancer cell sensitivity to trastuzumab. *Oncogene*. 2021 May;40(21):3719-3733.
- [15] Very N, Lefebvre T, El Yazidi-Belkoura I. Drug resistance related to aberrant glycosylation in colorectal cancer. *Oncotarget*. 2018 Jan 2;9(1):1380-1402.
- [16] Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015 Sep;15(9):540-55.
- [17] Reis CA, Osorio H, Silva L, et al. Alterations in glycosylation as biomarkers for cancer detection. *J Clin Pathol*. 2010 Apr;63(4):322-9.
- [18] Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun Signal*. 2008 Dec 4;6:10.
- [19] Trinchera M, Aronica A, Dall'Olio F. Selectin Ligands Sialyl-Lewis a and Sialyl-Lewis x in Gastrointestinal Cancers. *Biology (Basel)*. 2017 Feb 23;6(1).
- [20] Gomes C, Osorio H, Pinto MT, et al. Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS One*. 2013 Jun;8(6):e66737.
- [21] Gomes C, Almeida A, Barreira A, et al. Carcinoembryonic antigen carrying SLe(X) as a new biomarker of more aggressive gastric carcinomas. *Theranostics*. 2019 Oct;9(24):7431-7446.
- [22] Yin X, Rana K, Ponmudi V, et al. Knockdown of fucosyltransferase III disrupts the adhesion of circulating cancer cells to E-selectin without affecting hematopoietic cell adhesion. *Carbohydr Res*. 2010 Nov 2;345(16):2334-42.
- [23] Guerrero PE, Miro L, Wong BS, et al. Knockdown of alpha2,3-Sialyltransferases Impairs Pancreatic Cancer Cell Migration, Invasion and E-selectin-Dependent Adhesion. *Int J Mol Sci*. 2020 Aug 28;21(17).
- [24] Sakuma K, Aoki M, Kannagi R. Transcription factors c-Myc and CDX2 mediate E-selectin ligand expression in colon cancer cells undergoing EGF/bFGF-induced epithelial-mesenchymal transition. *Proc Natl Acad Sci U S A*. 2012 May 15;109(20):7776-81.
- [25] Carvalho AS, Harduin-Lepers A, Magalhaes A, et al. Differential expression of alpha-2,3-sialyltransferases and alpha-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. *Int J Biochem Cell Biol*. 2010 Jan;42(1):80-9.
- [26] Perez-Garay M, Arteta B, Pages L, et al. alpha2,3-sialyltransferase ST3Gal III modulates pancreatic cancer cell motility and adhesion in vitro and enhances its metastatic potential in vivo. *PLoS One*. 2010 Sep 1;5(9).

- [27] Wang D, Madunic K, Zhang T, et al. High Diversity of Glycosphingolipid Glycans of Colorectal Cancer Cell Lines Reflects the Cellular Differentiation Phenotype. *Mol Cell Proteomics*. 2022 Jun;21(6):100239.
- [28] Costa AF, Campos D, Reis CA, et al. Targeting Glycosylation: A New Road for Cancer Drug Discovery. *Trends Cancer*. 2020 Sep;6(9):757-766.
- [29] Charvat RA, Arrizabalaga G. Oxidative stress generated during monensin treatment contributes to altered *Toxoplasma gondii* mitochondrial function. *Sci Rep*. 2016 Mar 15;6:22997.
- [30] Deng Y, Zhang J, Wang Z, et al. Antibiotic monensin synergizes with EGFR inhibitors and oxaliplatin to suppress the proliferation of human ovarian cancer cells. *Scientific Reports*. 2015 Dec;5(1):17523.
- [31] Iljin K, Ketola K, Vainio P, et al. High-Throughput Cell-Based Screening of 4910 Known Drugs and Drug-like Small Molecules Identifies Disulfiram as an Inhibitor of Prostate Cancer Cell Growth. *Clinical Cancer Research*. 2009 Oct;15(19):6070-6078.
- [32] Ketola K, Vainio P, Fey V, et al. Monensin Is a Potent Inducer of Oxidative Stress and Inhibitor of Androgen Signaling Leading to Apoptosis in Prostate Cancer Cells. *Molecular Cancer Therapeutics*. 2010 Dec;9(12):3175-3185.
- [33] Mottola-Hartshorn C, Lorenzoni P, Ceccarini C. Biosynthesis and glycosylation of the carcinoembryonic antigen. *Biochem J*. 1988 Nov 1;255(3):943-8.
- [34] Secme M, Kocoglu SS. Investigation of the TLR4 and IRF3 signaling pathway-mediated effects of monensin in colorectal cancer cells. *Med Oncol*. 2023 May 23;40(7):187.
- [35] Tumova L, Pombinho AR, Vojtechova M, et al. Monensin inhibits canonical Wnt signaling in human colorectal cancer cells and suppresses tumor growth in multiple intestinal neoplasia mice. *Mol Cancer Ther*. 2014 Apr;13(4):812-22.
- [36] Vanneste M, Huang Q, Li M, et al. High content screening identifies monensin as an EMT-selective cytotoxic compound. *Sci Rep*. 2019 Feb 4;9(1):1200.
- [37] Wang X, Wu X, Zhang Z, et al. Monensin inhibits cell proliferation and tumor growth of chemo-resistant pancreatic cancer cells by targeting the EGFR signaling pathway. *Scientific Reports*. 2018 Dec;8(1).
- [38] Xin H, Li J, Zhang H, et al. Monensin may inhibit melanoma by regulating the selection between differentiation and stemness of melanoma stem cells. *PeerJ*. 2019 Jul;7:e7354.
- [39] Zhou Y, Deng Y, Wang J, et al. Effect of antibiotic monensin on cell proliferation and IGF1R signaling pathway in human colorectal cancer cells. *Ann Med*. 2023 Dec;55(1):954-964.

- [40] Padua D, Barros R, Amaral AL, et al. A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin. *Cancers (Basel)*. 2020 Feb 20;12(2).
- [41] Mollenhauer HH, Rowe LD, Witzel DA. Effect of monensin on the morphology of mitochondria in rodent and equine striated muscle. *Vet Hum Toxicol*. 1984 Feb;26(1):15-9.
- [42] Lewis AL, Chen X, Schnaar RL, et al. Sialic Acids and Other Nonulosonic Acids. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology*. 4th ed. Cold Spring Harbor (NY)2022. p. 185-204.
- [43] Caldeira C, Neves WS, Cury PM, et al. Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am J Kidney Dis*. 2001 Nov;38(5):1108-12.
- [44] Kouyoumdjian JA, Morita MP, Sato AK, et al. Fatal rhabdomyolysis after acute sodium monensin (Rumensin) toxicity: case report. *Arq Neuropsiquiatr*. 2001 Sep;59(3-A):596-8.
- [45] Zhang Z, Cui S, Zhang J. Rhabdomyolysis and hepatotoxicity following accidental monensin ingestion: A report of two cases. *Toxicol Ind Health*. 2021 Jan;37(1):34-37.
- [46] Blain M, Garrard A, Poppenga R, et al. Survival After Severe Rhabdomyolysis Following Monensin Ingestion. *J Med Toxicol*. 2017 Sep;13(3):259-262.
- [47] Rossing E, Pijnenborg JFA, Boltje TJ. Chemical tools to track and perturb the expression of sialic acid and fucose monosaccharides. *Chem Commun (Camb)*. 2022 Nov 1;58(87):12139-12150.
- [48] Shabir G, Saeed A, Zahid W, et al. Chemistry and Pharmacology of Fluorinated Drugs Approved by the FDA (2016-2022). *Pharmaceuticals (Basel)*. 2023 Aug 15;16(8).
- [49] Rillahan CD, Antonopoulos A, Lefort CT, et al. Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. *Nat Chem Biol*. 2012 Jul;8(7):661-8.
- [50] Bull C, Boltje TJ, Wassink M, et al. Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. *Mol Cancer Ther*. 2013 Oct;12(10):1935-46.
- [51] Orozco-Moreno M, Visser EA, Hodgson K, et al. Targeting aberrant sialylation and fucosylation in prostate cancer cells using potent metabolic inhibitors. *Glycobiology*. 2023 Dec 30;33(12):1155-1171.
- [52] Carrascal MA, Silva M, Ramalho JS, et al. Inhibition of fucosylation in human invasive ductal carcinoma reduces E-selectin ligand expression, cell proliferation, and ERK1/2 and p38 MAPK activation. *Mol Oncol*. 2018 May;12(5):579-593.

- [53] van Wijk XM, Lawrence R, Thijssen VL, et al. A common sugar-nucleotide-mediated mechanism of inhibition of (glycosamino)glycan biosynthesis, as evidenced by 6F-GalNAc (Ac3). *FASEB J.* 2015 Jul;29(7):2993-3002.
- [54] Jackson EG, Cutolo G, Yang B, et al. 4-Deoxy-4-fluoro-GalNAz (4FGalNAz) Is a Metabolic Chemical Reporter of O-GlcNAc Modifications, Highlighting the Notable Substrate Flexibility of O-GlcNAc Transferase. *ACS Chem Biol.* 2022 Jan 21;17(1):159-170.
- [55] Del Solar V, Gupta R, Zhou Y, et al. Robustness in glycosylation systems: effect of modified monosaccharides, acceptor decoys and azido sugars on cellular nucleotide-sugar levels and pattern of N-linked glycosylation. *Mol Omics.* 2020 Aug 1;16(4):377-386.
- [56] Marathe DD, Buffone A, Jr., Chandrasekaran EV, et al. Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins. *Blood.* 2010 Feb 11;115(6):1303-12.
- [57] Pouilly S, Piller V, Piller F. Metabolic glycoengineering through the mammalian GalNAc salvage pathway. *FEBS J.* 2012 Feb;279(4):586-98.
- [58] Zaro BW, Yang YY, Hang HC, et al. Chemical reporters for fluorescent detection and identification of O-GlcNAc-modified proteins reveal glycosylation of the ubiquitin ligase NEDD4-1. *Proc Natl Acad Sci U S A.* 2011 May 17;108(20):8146-51.
- [59] Barthel SR, Antonopoulos A, Cedeno-Laurent F, et al. Peracetylated 4-fluoro-glucosamine reduces the content and repertoire of N- and O-glycans without direct incorporation. *J Biol Chem.* 2011 Jun 17;286(24):21717-31.
- [60] Du Z, Lovly CM. Mechanisms of receptor tyrosine kinase activation in cancer. *Mol Cancer.* 2018 Feb 19;17(1):58.
- [61] Ferreira IG, Pucci M, Venturi G, et al. Glycosylation as a Main Regulator of Growth and Death Factor Receptors Signaling. *Int J Mol Sci.* 2018 Feb 15;19(2).
- [62] Lopez-Sambrooks C, Shrimal S, Khodier C, et al. Oligosaccharyltransferase inhibition induces senescence in RTK-driven tumor cells. *Nat Chem Biol.* 2016 Dec;12(12):1023-1030.
- [63] de-Freitas-Junior JCM, Andrade-da-Costa J, Silva MC, et al. Glycans as Regulatory Elements of the Insulin/IGF System: Impact in Cancer Progression. *Int J Mol Sci.* 2017 Sep 7;18(9).
- [64] Ho WL, Chou CH, Jeng YM, et al. GALNT2 suppresses malignant phenotypes through IGF-1 receptor and predicts favorable prognosis in neuroblastoma. *Oncotarget.* 2014 Dec 15;5(23):12247-59.

- [65] Wang Y, Fukuda T, Isaji T, et al. Loss of alpha1,6-fucosyltransferase inhibits chemical-induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways. *FASEB J.* 2015 Aug;29(8):3217-27.
- [66] Lin MC, Huang MJ, Liu CH, et al. GALNT2 enhances migration and invasion of oral squamous cell carcinoma by regulating EGFR glycosylation and activity. *Oral Oncol.* 2014 May;50(5):478-84.
- [67] Lin TC, Chen ST, Huang MC, et al. GALNT6 expression enhances aggressive phenotypes of ovarian cancer cells by regulating EGFR activity. *Oncotarget.* 2017 Jun 27;8(26):42588-42601.
- [68] Wu YM, Liu CH, Huang MJ, et al. C1GALT1 enhances proliferation of hepatocellular carcinoma cells via modulating MET glycosylation and dimerization. *Cancer Res.* 2013 Sep 1;73(17):5580-90.
- [69] Duarte HO, Balmana M, Mereiter S, et al. Gastric Cancer Cell Glycosylation as a Modulator of the ErbB2 Oncogenic Receptor. *Int J Mol Sci.* 2017 Oct 28;18(11).
- [70] Macauley MS, Arlian BM, Rillahan CD, et al. Systemic blockade of sialylation in mice with a global inhibitor of sialyltransferases. *J Biol Chem.* 2014 Dec 19;289(51):35149-58.
- [71] Miro L, Lopez J, Guerrero PE, et al. Sialyltransferase Inhibitor Ac(5)3F(ax)Neu5Ac Reverts the Malignant Phenotype of Pancreatic Cancer Cells, and Reduces Tumor Volume and Favors T-Cell Infiltrates in Mice. *Cancers (Basel).* 2022 Dec 12;14(24).
- [72] Do KT, Chow LQM, Reckamp K, et al. First-In-Human, First-In-Class, Phase I Trial of the Fucosylation Inhibitor SGN-2FF in Patients with Advanced Solid Tumors. *Oncologist.* 2021 Nov;26(11):925-e1918.
- [73] Hornik S, Cervenková Stastná L, Curinová P, et al. Synthesis and in vitro cytotoxicity of acetylated 3-fluoro, 4-fluoro and 3,4-difluoro analogs of D-glucosamine and D-galactosamine. *Beilstein J Org Chem.* 2016 Apr;12:750-9.
- [74] Hamala V, Ondrášková K, Šťastná L, et al. Improving the anticancer activity of fluorinated glucosamine and galactosamine analogs by attachment of a ferrocene or ruthenium tetrazene motif. *Applied Organometallic Chemistry.* 2024 Feb;38(5):e7399.
- [75] Moons SJ, Hornikx D, Aasted MKM, et al. UV light-induced spatial loss of sialic acid capping using a photoactivatable sialyltransferase inhibitor. *RSC Chem Biol.* 2023 Jul 5;4(7):506-511.
- [76] Pijnenborg JFA, Visser EA, Noga M, et al. Cellular Fucosylation Inhibitors Based on Fluorinated Fucose-1-phosphates*. *Chemistry.* 2021 Feb 24;27(12):4022-4027.

- [77] Bull C, Boltje TJ, van Dinther EA, et al. Targeted delivery of a sialic acid-blocking glycomimetic to cancer cells inhibits metastatic spread. *ACS Nano*. 2015 Jan 27;9(1):733-45.
- [78] Surolia R, Pachauri M, Ghosh PC. Preparation and characterization of monensin loaded PLGA nanoparticles: in vitro anti-malarial activity against *Plasmodium falciparum*. *J Biomed Nanotechnol*. 2012 Feb;8(1):172-81.
- [79] Ferdous AJ, Stembridge NY, Singh M. Role of monensin PLGA polymer nanoparticles and liposomes as potentiator of ricin A immunotoxins in vitro. *J Control Release*. 1998 Jan 2;50(1-3):71-8.
- [80] Shaik MS, Ikediobi O, Turnage VD, et al. Long-circulating monensin nanoparticles for the potentiation of immunotoxin and anticancer drugs. *J Pharm Pharmacol*. 2001 May;53(5):617-27.
- [81] Xiao H, Woods EC, Vukojicic P, et al. Precision glycocalyx editing as a strategy for cancer immunotherapy. *Proc Natl Acad Sci U S A*. 2016 Sep 13;113(37):10304-9.
- [82] Sudhan Shaik M, Kanikkannan N, Singh M. Conjugation of anti-My9 antibody to stealth monensin liposomes and the effect of conjugated liposomes on the cytotoxicity of immunotoxin. *J Control Release*. 2001 Oct 19;76(3):285-95.
- [83] Fu Z, Li S, Han S, et al. Antibody drug conjugate: the "biological missile" for targeted cancer therapy. *Signal Transduct Target Ther*. 2022 Mar 22;7(1):93.
- [84] Tsuchikama K, Anami Y, Ha SYY, et al. Exploring the next generation of antibody-drug conjugates. *Nat Rev Clin Oncol*. 2024 Mar;21(3):203-223.

Appendix I

Novel anticancer drug discovery efforts targeting glycosylation: the emergence of fluorinated monosaccharides analogs

Ana F. Costa; Andreia Teixeira; Celso A. Reis and Catarina Gomes

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Novel anticancer drug discovery efforts targeting glycosylation: the emergence of fluorinated monosaccharides analogs

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ABSTRACT

Introduction: Glycosylation is an essential enzymatic process of building glycan structures that occur mainly within the cell and gives rise to a diversity of cell surface and secreted glycoconjugates. These glycoconjugates play vital roles, for instance in cell-cell adhesion, interaction and communication, activation of cell surface receptors, inflammatory response and immune recognition. This controlled and well-coordinated enzymatic process is altered in cancer, leading to the biosynthesis of cancer-associated glycans, which impact glycan-dependent biological roles.

Areas covered: In this review, the authors discuss the importance of targeting cancer-associated glycans through potent glycan biosynthesis inhibitors. It focuses on the use of analogs, providing an overview of findings involving these in cancer. The highly explored fluorinated monosaccharide analogs targeting aberrant glycosylation are described, aiming to inspire advances in the field.

Expert opinion: Altered glycosylation, such as increased sialylation and fucosylation, is a feature in cancer and has been shown to play key roles in several malignant properties of cancer cells. Strategies aiming at remodeling cancer cells' glycome are emerging and present a huge potential for cancer therapy. Fluorinated monosaccharides have been gathering promising preclinical results as novel cancer drugs. Nevertheless, cancer specific targeting strategies must be considered to avoid significant side effects.

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1. Introduction

Glycosylation is a cellular process that consists in the enzymatic building of carbohydrate moieties to central macromolecules such as proteins and lipids [1,2]. This complex process occurs essentially in the endoplasmic reticulum (ER) and Golgi apparatus of all living cells and involves the sequential and coordinated action of hundreds of glycosyltransferases (GTs) and glycosidases, giving rise to distinct glycoconjugates [2,3]. The generated glycoconjugates are then exported to the cell membrane or secreted in the extracellular space, playing crucial roles in various biological processes, including cell signaling, cell-cell interaction, and immune response [2,4]. Another type of glycosylation takes place in the cytoplasm and nucleus (GlcNAc-type O-glycosylation) and is responsible for regulating transcription, translation, and mitochondrial function [5].

Thus, any alterations that might occur throughout the glycosylation process, even if minor, significantly impact the cellular glycome and therefore its essential function, leading to the development of several pathologies [4,6].

1.1. Monosaccharides, the sugar substrates of glycosyltransferases

Apart from the hundreds of enzymes responsible for the biosynthesis of the complex and very diverse glycan structures,

glycosylation is also closely dependent on the ability of the cell to create GTs substrates: the sugar donors. Sugar donors or nucleotide sugars are activated forms of monosaccharides, the basic unit of glycan biosynthesis. Succinctly, sugar donors can be salvaged from recycled glycoconjugates by lysosomal glycosidases or converted enzymatically at the cytoplasm mainly from imported glucose, galactose and fructose residues. The derived monosaccharides are then activated to high-energy sugars through many enzymatic steps, culminating in nucleotide addition, commonly of UDP, GDP, or CMP. These activated sugars are then transported through nucleotide sugar transporters into the Golgi and the ER, where they serve as glycans building block units for glycan biosynthesis in cells [7]. Furthermore, in addition to the ER and Golgi, transporters present on the cell surface and lysosomes ensure the proper trafficking and availability of sugar donors for glycan synthesis, playing an essential role throughout the glycosylation process [7] (Figure 1).

1.2. Glycan biosynthesis in cancer

Alterations in the normal glycosylation pattern have been widely observed in cancer cells and are considered a feature of malignant transformation and progression [3,6]. This primarily arises due to deregulations in the various glycan biosynthetic pathways, including the altered expression, activity

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Article highlights

- Glycosylation is a molecular process responsible for the synthesis glycoconjugates that play vital roles in cellular biology.
- Monosaccharide sugar donors, the basic unit for glycans assembly, are acquired by the conversion of imported glucose, galactose and/or fructose within the cell or through a salvage pathway from glycoconjugates at the lysosome.
- Alterations in the normal process of glycosylation are commonly found in cancer. These changes are reported as playing key roles in cancer progression.
- Targeting the biosynthesis of cancer-associated glycans constitutes a potential strategy for novel cancer drug development;
- Saccharide analogs have been explored in the cancer drug field.
- Distinct fluorinated monosaccharide analogs have shown promising *in vitro* and *in vivo* results in cancer research.

or localization of GTs and/or glycosidases [4,8], alteration of nucleotide sugar transporters [9,10] and also altered expression of key GTs chaperones [3,11]. Furthermore, low or lack of availability of sugar nucleotide donors, and an improper function of the ER and Golgi apparatus can also affect glycan biosynthesis [6,12,13].

The expression of several glycan alterations in cancer has been largely well-documented. These major modifications include the biosynthesis of truncated O-glycans (such as the Tn and Sialyl Tn antigens), changes in N-glycans, with increased branched structures, and increased sialylation and/or fucosylation. These modifications lead to the appearance of the sialyl Lewis's antigens on glycolipids and glycoproteins, which are some of the common modifications observed in cancer [3,6] (Figure 2). Such glycan alterations impact a wide range of key biological processes, including neoplastic cell survival and cancer cell growth, angiogenesis, cell adhesion, migration and invasion, and consequently metastasis formation, escape mechanisms to the immune system, as well as therapy resistance [3,4]. Therefore, targeting glycosylation pathways has emerged as a promising strategy for cancer therapy [14–17].

Next in this review, we will focus on the recent strategies that modulate the expression of altered glycan in cancer. We will discuss current data revolving around the most exploited Fluorinated Monosaccharide Analogs (F-MAs) as glycan biosynthesis inhibitors to fight aberrant glycans in cancer and hope to inspire new efforts to advance progress in this promising field.

2. Altered glycosylation: a promising target in cancer

Nowadays, cancer patients' treatment options include surgery, once possible, and chemotherapy and/or radiotherapy. When eligible and available, precise therapies directed to cancer cells' specific receptors as well as immunotherapy and small molecule inhibitors have also been incorporated into clinical therapeutic procedures. Especially in several advanced tumor stages, specific molecular targeting approaches are invaluable for use in clinical practice [18]. Small molecules, mostly inhibitors of receptor kinases, and monoclonal antibodies (mAbs)

are currently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), with several other ongoing clinical trials [19]. Although beneficial for the treatment of cancer patients, these therapies have encountered some limitations, such as being restricted to a small subset of cancer patients, the associated toxic side effects, and the emergence of therapy resistance [19,20]. Thus, the need for alternative novel and unexplored targets, such as cancer-associated glycans, are of major interest holding potential as novel cancer therapies.

Due to their important role in several aspects of cancer development and progression, glycans and their biosynthetic pathways are emerging as potential targets for the development of effective therapies. The generation of vaccines and antibodies directed to cancer-associated glycans and glycoproteins have largely been studied, representing some of the first attempts to target glycans in cancer therapy [21–23]. This field of research has provided the basis for recent advanced pre-clinical and clinical studies, being therapeutic options in several types of neoplasia [24–26]. The use of sialidases, enzymes that remove sialic acids from glycan structures, was also among the initial strategies proposed to address aberrant sialylation in cancer cells [27]. Although bacterial sialidases showed promising results, their labor-intensive and cost-ineffective purification process, coupled with short-term depletion of cell surface sialic acids, limited their therapeutic potential [28,29].

Besides the above-mentioned strategies, the search for glycosylation inhibitors is emerging as a novel approach to find new cancer drugs. Usually, the search for novel inhibitor drugs is conducted through high throughput screening (HTS) analysis of libraries comprising thousands of compounds [14]. However, regarding glycosylation inhibitors, few reports are available until date focusing on the identification of novel glycan inhibitors by HTS, either by targeting GTs or cancer-associated glycans [30–32]. Indeed, compared to other molecular targets, these analyses are still in a small number and lack further validation and clinical studies to be considered therapeutic possibilities [14].

Alternatively to this, recently, strategies using chemical synthesis of glycosylation inhibitors, commonly monosaccharide analogs that interfere with the biosynthesis of aberrant glycans present in tumor cells, are emerging as promising approaches [15,17,33–35]. Specifically, through the chemical introduction of functional groups on the common monosaccharides, these molecules are recognized by the cell as the natural sugar, despite blocking the enzymatic addition of the natural monosaccharides to the glycan chain or impairing glycans elongation, thus inhibiting glycosylation pathways [33,36]. Over the years, several modifications and functional groups were introduced into different monosaccharides, including fluorine [33], azide [37], alkynyl [38], or thioester [39]. Among the many options overall these analogs achieved very promising results in blocking certain glycan structures and in tackling cancer cell malignancy.

Particularly, the introduction of one or more fluorine groups is a very common monosaccharide modification. In fact, over the past 20 years, fluorinated drugs have become one of the fastest-growing classes of organic compounds.

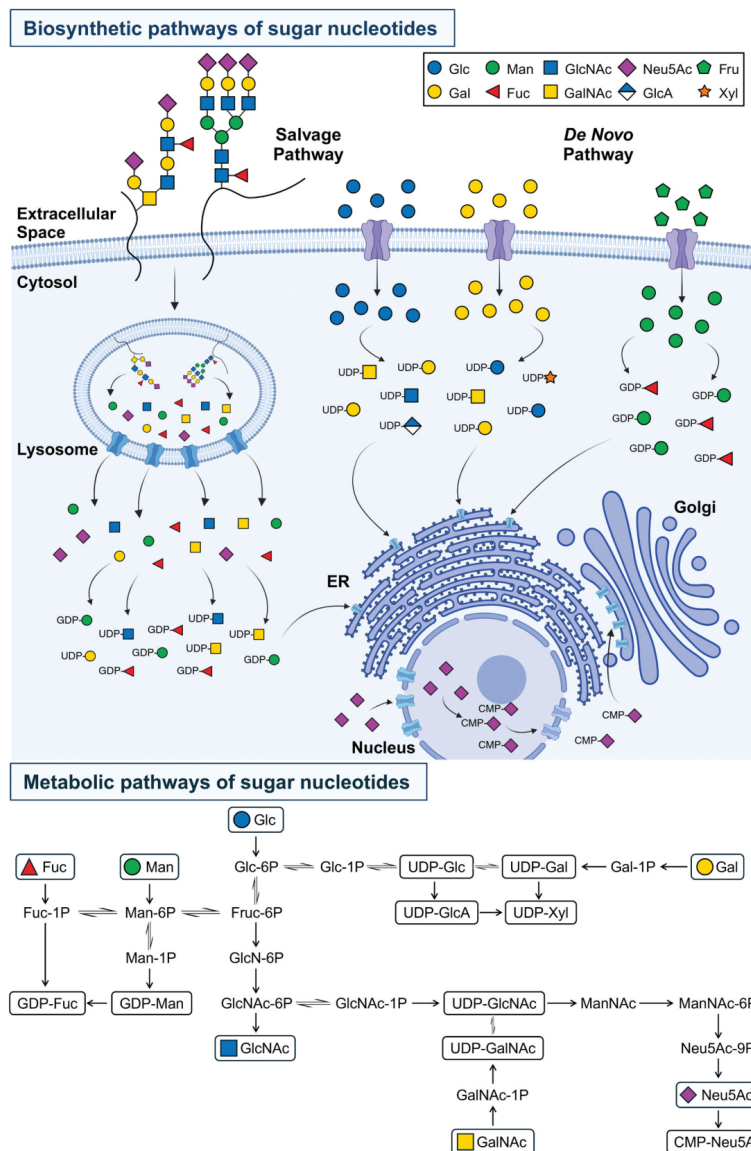


Figure 1. Schematic representation of the biosynthetic and metabolic pathways involved in the synthesis of sugar nucleotide donors. Monosaccharides are derived from glycans released from degraded glycoproteins at lysosomes (salvage pathway) or from free residues available at the cell's cytosol resulting from the enzymatic conversion of imported glucose and/or fructose (*de novo* pathway). Activated forms of the monosaccharides or sugar donors are acquired by enzymatic addition of nucleotides, commonly UDP, GDP, or CMP, to the sugars.

Created with *BioRender.com*. Legend: CMP – cytidine monophosphate, Fuc – Fucose, Gal – Galactose, GalNAc – N-Acetylgalactosamine, GDP – Guanosine diphosphate, Glc – Glucose, GlcA – Glucuronic Acid, GlcNAc – N-Acetylglucosamine, Man – Mannose, Neu5Ac – N-acetylneuraminic acid, UDP – Uridine diphosphate, Xyl – Xylose.

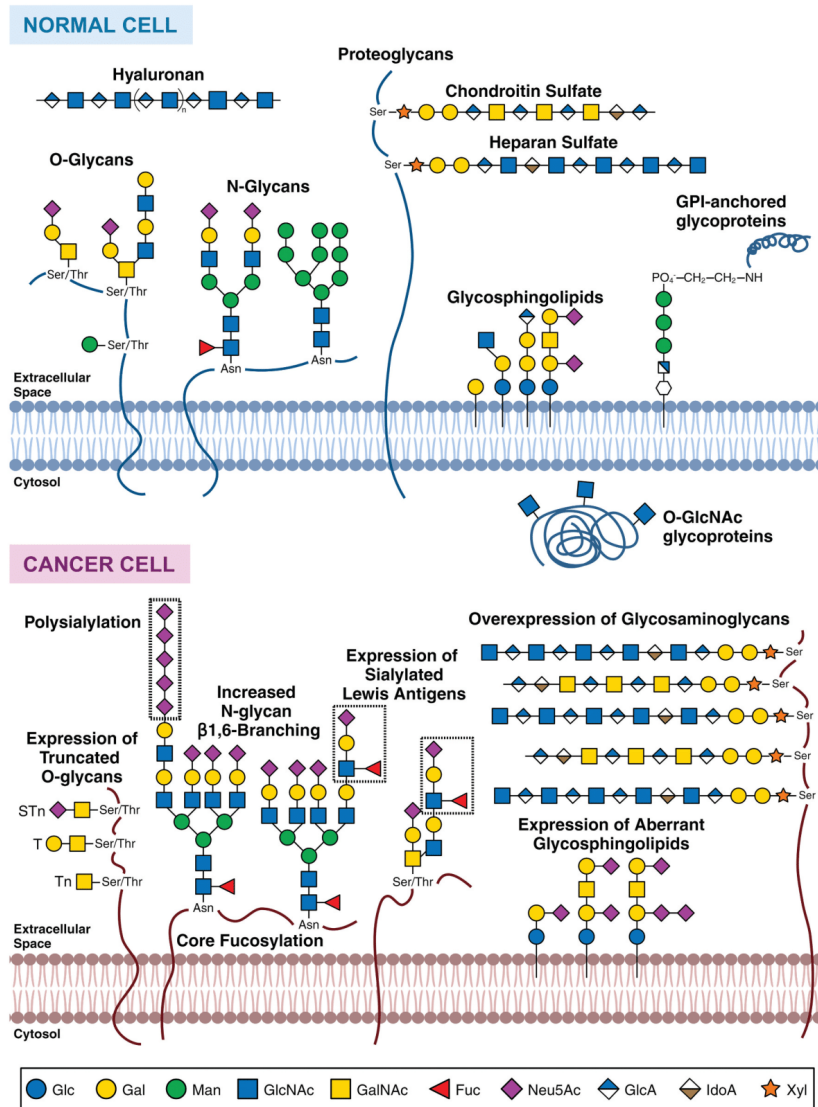


Figure 2. Schematic representation of some glycoconjugates found at the surface of normal and cancer human cells. According to the molecule to which glycans are attached, several classes of glycoconjugates are defined, including glycoproteins (in which glycans are attached to asparagine (N-glycans) or serine/threonine (O-glycans) amino acids), proteoglycans (where long glycosaminoglycan (GAG) chains are attached to proteins), glycosylphosphatidylinositol (GPI)-anchored glycoproteins (in which a protein is attached to a GPI structure that, in turn, is attached to membrane lipids) and glycosphingolipids (whose glycans are attached to lipids). Altered glycosylation is a feature in cancer, and the most reported cancer-associated glycans are the expression of truncated O-glycan structures, the aberrant sialylation of glycosphingolipids, as well as increased sialylation of glycoproteins (through both polysialylation and expression of sialylated Lewis glycans), core fucosylation, and β 1,6-branching of N-glycans.

Created with BioRender.com. Legend: Fuc – Fucose, Gal – Galactose, GalNAc – N-Acetylgalactosamine, Glc – Glucose, GlcA – Glucuronic Acid, GlcNAc – N-Acetylglucosamine, IdoA – Iduronic Acid, Man – Mannose, Neu5Ac – N-acetylneuraminic acid, Xyl – Xylose.

These pharmaceuticals have made up approximately 20% of commercialized medications nowadays [40]. In glycoscience, fluorine was first employed in probes to study glycan–protein interaction, specifically by using ^{18}F as a radiolabel tracer atom for positron emission tomography (PET) imaging [41]. In addition, 2-Deoxy-2- ^{18}F -fluoro-D-glucose is the most commonly used PET tracer in cancer diagnosis applications [42].

The synthesis of F-MAs involves the chemical replacement of one or more hydroxyl groups (–OH) attached to the sugar carbons of natural monosaccharides with fluorine atoms (deoxyfluorination). The first descriptions of this substitution began in the last century and continued through the years until today [33,43–45]. Fluorine is a small size hydrophobic atom, chemically inert, and ideal for translational studies. It is commonly employed as bioisosteres of the OH- and H-groups, due to its polarity and similarity in size. Having a significant electronegativity, this atom destabilizes the oxocarbenium transition state in the reactions catalyzed by carbohydrate-binding enzymes, altering the enzyme activity [41]. Nevertheless, the main challenge of using fluorinated analogs in biological contexts is ensuring the efficient crossing of the cell membranes, which represents an important limitation of the drug's efficiency in living cells [33]. This hurdle can be overcome by peracetylation of the F-MAs, which was described as improving cell permeability while also being easily and rapidly converted into the corresponding donor substrate within the cell [33,46]. Indeed, distinct peracetylated F-MAs were tested in several cancer models (Table 1), as described in the following sections.

2.1. Fluorinated N-acetylglucosamine (F-GlcNAc)

Analogues of N-acetylglucosamine (GlcNAc) were among the first to be synthesized. During the '90s, 4 F-GlcNAc was described by Woynarowska and colleagues as a glycan inhibitor, reducing the expression of terminal lactosamine, Sialyl Lewis X (SLeX) and Sialyl Lewis A (SLeA) structures and hindering cell adhesion in ovarian and colon cancer cell lines [44,47]. Peracetylated 4 F-GlcNAc was later shown to impair the expression of selectin ligands such SLeX, and inhibit cell adhesion in leukemia cancer cells. In the same study, it was shown that this analog is not incorporated into N- or O-glycans. Instead, after entering the cell, 4 F-GlcNAc is readily metabolized into UDP-4 F-GlcNAc, thereby decreasing the cellular amounts of the natural nucleotide sugar UDP-GlcNAc and UDP-GalNAc through a negative feedback loop [48]. In a mouse model of attenuate skin inflammatory reaction, peracetylated 4 F-GlcNAc also led to a reduction of E- and P-selectin ligand synthesis and prevented leukocyte migration [49]. Additionally, intratumoral administration of 4 F-GlcNAc attenuated tumor growth in mice by inhibition of galectin-1 ligand synthesis and galectin-1 mediated tumor immune evasion. In fact, it was demonstrated that 4 F-GlcNAc treatment led to an increase of effector T cells and a decrease of interleukin-10 positive (IL-10^+) T cells in galectin-1 expressing tumors, hence it was proposed that the 4 F-GlcNAc could limit tumor growth via enhancing T cell-mediated immunity [50,51]. Moreover, 4 F-GlcNAc was also shown to inhibit protein O-GlcNAc levels, similar to peracetylated 4 F- and 6 F-GalNAc [52].

2.2. Fluorinated N-acetylgalactosamine (F-GalNAc)

Distinct fluorinated GalNAc analogs have been showing the capacity to impair glycans biosynthesis and their associated biological roles. For instance, human leucocyte cells treated with peracetylated 4 F-GalNAc showed a reduced leukocyte selectin-mediated adhesion and migration to sites of inflammation due to SLeX inhibition. Additionally, it has been shown that 4 F-GalNAc can be incorporated into glycans structures of multiple cellular glycoproteins, including P-selectin glycoprotein ligand-1 (PSGL-1), impairing the normal glycosylation of these proteins [53]. However, other studies suggested that UDP-4 F-GalNAc acts by inhibiting the production of endogenous UDP-GalNAc/GlcNAc [54], and also by inducing a slight decrease of CMP-Neu5Ac and GDP-Man [37], further empathizing their role in sugar donor depletion.

Further modifications of 4 F-GalNAc, as well as to 3F-GalNAc structure, such as 1-O-deacetylation, showed an increased efficacy in inhibiting the proliferation of human prostate cancer cells, even when compared to common chemotherapeutic agents, cisplatin and 5-fluorouracil [55]. The chemical addition of an organometallic moiety at the C2 position of 4 F-GalNAc also showed an increased cytotoxicity against cisplatin-resistant breast and ovarian cancer cells [56].

Apart from N- and O-glycans, peracetylated 4 F-GalNAc also affects glycosaminoglycan (GAG) biosynthesis, except for Heparan sulfate structures. Authors suggested that this could be due to the fact that the carbon 4 position, where the fluorine atom is added, is directly involved in the conversion of UDP-GalNAc into UDP-GlcNAc and vice versa, hindering the epimerization process by the UDP-glucose 4-epimerase enzyme [52]. On the other hand, peracetylated 6 F-GalNAc drastically reduces all GAGs expression (chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS)), as well as of N- and O-linked glycans, since the modified position is not directly involved in either of these processes. Therefore, there is a strong reduction of cellular UDP-GalNAc and UDP-GlcNAc. Moreover, 6 F-GalNAc treatment, using an *in vivo* Chick embryo chorioallantoic membrane (CAM) model, was shown to decrease growth factor signaling and angiogenesis [52].

2.3. Fluorinated sialic acid (F-Neu5Ac)

In 2012, it was described a global inhibitor of sialyltransferases, the peracetylated 3F_{ax}-Neu5Ac. These authors showed that 3F_{ax}-Neu5Ac easily enters the cells and is converted into its fluorinated donor substrate analog CMP-3F_{ax}-Neu5Ac. Through a dual-action mechanism, it blocks enzymatic activity by competition with the natural Neu5Ac and accumulates within the cell which, due to the structural similarity to the natural sugar substrates, shuts down the *de novo* synthesis of this cell substrate through a feedback loop, leading to a more prolonged effect [33]. Using leukemia cells, authors also showed that 3F_{ax}-Neu5Ac successfully inhibited several sialyltransferases, such as ST3GalII/IV/VI and ST6Gal enzymes, resulting in a remarkable reduction of the general sialylation [33].

Biologically, 3F_{ax}-Neu5Ac efficiently inhibited adhesion and migration in murine melanoma cells, showing no major

Table 1. Overview of fluorinated monosaccharide analogs (F-MAs) used in cancer research.

F-Mas	Structure	Glycans affected	Cancer cells tested	Refs
4F-GlcNAc		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction of LacNAc structures, SLeX, SLeA and O-GlcNAc levels; - Inhibition of GAG biosynthesis: HS, DS and CS	- Ovarian - Colon - Melanoma - Leukemia	[44,47,48,50,52]
4F-GalNAc		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction of LacNAc structures, SLeX, and O-GlcNAc levels; - Inhibition of GAG biosynthesis: DS and CS	- Ovarian - Leukemia - Breast	[52,53]
6F-GalNAc		- Effect in <i>N</i>- and <i>O</i>-linked glycan biosynthesis; - Reduction O-GlcNAc levels; - Inhibition of GAG biosynthesis: HS, DS and CS	Ovarian	[52]
3F _{ax} -Neu5Ac		- Reduction of the general sialylation; - Increasing of overall fucosylation; - Abolishment of SLeX; - Reduction of Sialyl T antigen.	- Melanoma - Leukemia - Neuroblastoma - Cervical - Prostate - Pancreatic	[33,57,62,63,66,68]
3F _{ax} -Neu5Ac-5-carbamate		- More potent than 3F_{ax}-Neu5Ac; - Reduction of the general sialylation.	- Melanoma - Leukemia - Neuroblastoma - Cervical - Prostate	[45,68]
2F-Fuc		- Reduction of the general fucosylation; - Reduction of core fucosylation; - Abolishment of SLeX and LeX.	- Leukemia - Liver - Colon - Cervical - Lung - Prostate	[45,70–72,74,78]
2F-Fuc-1-P		- More potent than 2 F-Fuc; - Reduction of the general fucosylation.	- Leukemia - Cervical - Lung - Prostate	[45,74]
Fucotrim I		- More potent than 2 F-Fuc; - Reduction of the general fucosylation; - Increase of α2,6- and α2,3-linked sialic acids	- Leukemia - Prostate	[45,78]
Fucotrim II		- More potent than 2 F-Fuc; - Reduction of the general fucosylation; - Reduction of mannose N-glycans and α2,6- and α2,3-linked sialic acids;	- Leukemia - Prostate	[45,78]

cellular toxicity and strongly inhibiting tumor growth *in vivo* [57]. Moreover, the treatment affected the rolling in E-selectin and P-selectin coated surfaces, due to the abolishment of SLeX expression in leukocytes and melanoma cells [33]. The biological interaction between selectins expressed in the endothelium and SLeX on leukocytes is essential in inflammatory stimulus-response, a process mimicked by cancer cells during metastasis formation [58–61]. In pancreatic cancer cells, a reduction in tumor sialylation, E-selectin adhesion, migration, and invasion were also observed by treating cells with 3F_{ax}-Neu5Ac [62].

In vivo intratumorally injection of 3F_{ax}-Neu5Ac led to a reduction of tumor growth and increased tumor-infiltrating lymphocytes [62,63]. Moreover, pre-treatment of prostate cancer cells with this inhibitor inhibited prostate tumor growth and spread to bones in mouse models [64]. In fact, 3F_{ax}-Neu5Ac had a major influence on the tumor microenvironment and tumor immunity by enhancing both tumor-infiltrating natural killer and CD8⁺

T-cells and reducing regulatory T-cell and myeloid regulatory cell numbers [63]. *In vivo* systemic administration of this analog led to a long-lasting decrease in sialic acid expression in various mouse tissues, although it was noticed a cell-type differential sensitivity. The inhibitory effect is most noticeable in metabolically active tissues, such as the liver. However, despite the good results in abolishing the increased sialylation in cancer cells, the use of 3F_{ax}-Neu5Ac *in vivo* has limitations, causing mice renal failure [65]. To overcome this, Büll and colleagues developed a targeted delivery system by encapsulating the 3F_{ax}-Neu5Ac into tumor-targeting nanoparticles. Therefore, this sialic acid-blocking analog is specifically delivered to melanoma cells *in vivo*, preventing the outgrowth of melanoma cells at distant sites while also limiting exposure to normal cells [66]. Very recently, another alternative was reported, with the synthesis of a photoactivatable 3F_{ax}-Neu5Ac derivative by adding a photolabile ortho-nitrobenzyl group for inducible and local inhibition of sialoglycan biosynthesis in target cells or tissues, reducing

damage in healthy tissues [67]. Further modifications of 3F_{ax}-Neu5Ac, by adding a carbamate residue into the C5 position, were also reported and demonstrated to be efficiently metabolized inside the cells, achieving higher effective inhibitor capacity. Consequently, an enhanced and prolonged inhibitory activity was observed in both mouse and human cancer and non-cancer cell lines upon the addition of this carbamate group [45,68].

2.4. Fluorinated fucose (F-Fuc)

The 2 F-fucose (2 F-Fuc) was first synthesized by Murray and colleagues in 1997. It is transported through the cell membrane and then converted to its nucleotide sugar analog, the GDP-2 F-Fuc. The GDP-2 F-Fuc was described to inhibit fucosylation through feedback inhibition of the native GDP-fucose biosynthesis pathway and also by competitive inhibition of fucosyltransferases [69]. In 2012, Paulson's lab demonstrated that the newly peracetylated 2 F-Fuc was efficient in inhibiting fucosyltransferase activity by targeting FUTIV, VII, and VIII enzymes, leading to the complete abolishment of SLeX and LeX and also to a reduction in core fucosylation [33]. *In vitro* and *in vivo* studies showed promising anti-cancer effects of the orally bioavailable 2 F-Fuc in several types of tumors [33,70–72]. In fact, 2 F-Fuc reached a phase I clinical trial (NCT 02952989) for patients with advanced solid tumors. In this trial, the potential of oral administration of 2 F-Fuc in suppressing fucosylation and tumor growth was demonstrated, although its use was associated with thromboembolic events leading to trial termination [73].

Due to their efficacy and promise as a cancer therapeutic, 2 F-Fuc has inspired the development of several additional inhibitors [36]. For example, the establishment of a 2 F-Fuc phosphate derivative, by adding a phosphate group at the C1 position, also showed an increased potency and prolonged inhibitory effect compared to the initial 2 F-Fuc [45,74]. Moreover, multi-fluorination at the C6 position, such as 6(2) F-Fuc and 6(3)F-Fuc (Fucostatin I) and its phosphate derivative Fucostatin II showed potent inhibitory activity against the proliferation of HCT116 colon cancer cell line and the angiogenic HUVEC cell line. However, these are less potent inhibitors of FUTVIII core fucosylation compared to 2 F-Fuc [75–77].

Inhibitors derived from fluorinated rhamnose 1-phosphate (Fucotrim I and II) can also effectively reduce cellular fucosylation [78]. These are converted into GDP-mannose derivatives within the cell, reducing the levels of GDP-mannose levels and inhibiting, by competition, the enzyme GDP-mannose dehydratase (GMDS), which is crucial for the *de novo* biosynthesis of GDP-fucose. Fucotrim I and II treatment inhibited effectively fucosylated glycans on the cell surface, cell proliferation, viability, and colony formation in prostate cancer cells without affecting normal prostate epithelium cells [45,78].

3. Conclusion

In summary, this review highlights the crucial role of glycosylation in cancer development and progression, emphasizing how targeting glycans holds therapeutic potential for the development of new drugs. Among several existing strategies for targeting cancer-associated glycosylation, the use of F-MAs

stands out for its promising results in both preclinical and clinical studies. F-MAs can effectively disrupt glycosylation pathways and, consequently, inhibit tumor growth, metastasis, and immune evasion, making them potential drugs for cancer therapy. Nonetheless, challenges, such as improving efficacy, and reducing off-target effects still need to be tackled. Thus, future research into the development and refinement of F-MAs compounds could lead to more effective cancer therapies.

4. Expert opinion

The role of glycan alterations in cancer development and progression is well recognized and known to affect many important molecular and cellular processes. Nevertheless, limited advances were made regarding drug development targeting glycan biosynthesis as molecular targets.

Deciphering glycans biosynthesis and possible biological roles is a hurdle that poses many distinct challenges. In fact, glycosylation is often simplified as an enzymatic molecular process that occurs mainly at the ER and at the Golgi. However, glycosylation is much more complex, being highly regulated process that involves hundreds of distinct enzymes with specific enzymatic activities and with regulated ER and Golgi locations. Additionally, the complexity of the glycosylation field is also enhanced by several other aspects such as: i) the enzymatic conversion of sugar donors and their transport to the ER and to the Golgi, ii) the impact of morphological and dynamic alterations at the ER and Golgi compartments, such as the ones that occur during cell cycle, iii) GTs enzymatic activity and redundancies, and iv) the concentration, availability and biological features of molecular acceptor substrates (glycan carriers), i.e. the glycoconjugates. Besides, there is a deficit of advanced tools and methodologies for glycan analysis at the cellular level, and, therefore, at more complex organs and disease settings, hindering the better understanding and dissection of glycosylation. In the last two decades, significant progression was made, specially by the development of distinct protocols based on mass spectrometry analysis to in-deep characterize glycan structures of different biological samples such as cells, fluids and tissues [79–84]. Additionally, the development of robust *in vitro* cellular models with new genetically engineered methodologies, such as CRISPR/Cas9, to glycoengineered several cell lines boosted the understanding of GTs functions, and importantly helped to decipher many glycan-mediated interactions and biological roles [85–95].

Despite all the encountered difficulties in clearly defining glycan structures, GTs activities, and glycoconjugate biological roles, for decades alterations in the normal glycosylation of cells have been massively identified. For instance, the high expression of sialylated glycans has been extensively documented and demonstrated to be associated with an increase in tumor cell growth and aggressiveness, resistance to apoptosis and immune evasion, resulting in metastasis formation and a poor prognosis for the patients [3,4,96–98]. Additionally, increased expression of fucosylated antigens is a common alteration in cancer, being associated with increased cell survival and proliferation, drug resistance to chemotherapy,

interaction with immune cells and cancer cell metastasis [4,99,100]. Therefore, interfering with the expression of cancer-associated glycans in cancer cells constitutes a therapeutic option to prevent cancer progression and metastasis formation. Accordingly, some strategies have been developed to target these glycans and associated glycosylation pathways.

In this review, we discuss the use of monosaccharide analogs, with particular focus on the emergent role of F-MAs as novel inhibitors of cancer-related biosynthetic pathways and, consequently, of their associated malignant properties. Further chemical modifications, such as the addition of phosphate and carbamate groups, were also discussed as methods to potentiate the inhibitory effects, hence leading to a more effective impairment of cancer malignancy [45,68,74]. Overall, F-Neu5Ac and F-Fucose analogs are the most studied F-MAs, showing already promising *in vivo* results, like the 3F_{ax}-Neu5Ac or the 2 F-Fuc that have recently entered a clinical trial [73]. It is important to note, however, that obstacles might be faced. Like in many other drugs, it is challenging to specifically tackle cancer cells without affecting normal cells. This is the case of 2 F-Fuc which failed due to patient safety issues [73] as well as 3F_{ax}-Neu5Ac which showed renal complications in pre-clinical studies in mice [65].

Taking this into consideration, recent reports already account for this specific cell-targeting obstacle. A targeted delivery system where 3F_{ax}-Neu5Ac was encapsulated was already established and showed good efficacy in reaching cancer cells and in preventing metastasis outgrowth, with neglected effects on normal cells [66]. In addition, the chemical addition of photo-active groups to the general 3F_{ax}-Neu5Ac formula was already tested. Results nicely showed that 3F_{ax}-Neu5Ac was biologically active only in irradiated sites and, consequently, these cells presented impaired sialylation [67]. This is a very promising result, yet its applicability may be limited to easily irradiated tissues/organs.

Additionally, to these approaches, we envision that the establishment of antibody-drug conjugates (ADCs) coupled with promising analogs targeting cancer-specific antigens can be a clever strategy to avoid side effects. Indeed, ADCs offer an innovative approach by combining the specificity of monoclonal antibodies with the efficacy of promising agents, mirroring first-line cancer therapies. Besides, ADCs harbor the potential to minimize toxicity by selectively delivering the payload to the tumor site. The promise of ADCs in targeting glycans was initially shown by Bertozzi's lab. Briefly, sialidases were linked with antibodies that specifically recognize a cancer cell target (Her2), stripping sialic acid cancer glycocalyx and, consequently, promoting a killing immune response. They also showed that this way of editing the cancer cell glycocalyx represents a promising approach to cancer immune therapy *in vivo* [27]. Despite challenges such as choosing suitable linkers, payloads, and ensuring targeted delivery to cancer cells to minimize side effects such as liver toxicity, several ADCs are making significant progresses [101,102]. These advancements demonstrate the potential of ADCs in cancer therapy despite the complexities involved. Important to mention that are also glycan mimetics, which have been used to target glycan-dependent interactions such as immune checkpoints in cancer and have been gathering encouraging results and should be considered in these

modalities [15,63,103,104]. In addition, the delivery of F-MAs to cancer cells has the potential to improve the effectiveness of other approved treatments. For instance, editing the cancer cell glycocalyx with specific F-MAs could improve the binding of some clinically approved mAbs.

Overall, efforts have been made toward the development of novel cancer therapies targeting the cancer glycome. Due to its critical role in many aspects of cancer development and progression, we believe these novel strategies will transform cancer therapy toward better patient outcomes. Nevertheless, the key steps for a successful application in oncology will depend on the right balance between specificity and efficiency of glycan biosynthesis inhibition in cancer cells. Moreover, it will require demonstration of pharmacodynamic features in which cancer-specific targeting strategies are efficiently and safely implemented.

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References

Papers of special note have been highlighted as either of interest (+) or of considerable interest (++) to readers.

1. Varki A. Biological roles of glycans. *Glycobiology*. 2017 Jan;27(1):3–49.
2. Schjoldager KT, Narimatsu Y, Joshi HJ, et al. Global view of human protein glycosylation pathways and functions. *Nat Rev Mol Cell Biol*. 2020 Dec;21(12):729–749. doi: 10.1038/s41580-020-00294-x
3. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015 Sep;15(9):540–555. doi:10.1038/nrc3982
- ++ An extensive review focusing on glycosylation in cancer.
4. Mereiter S, Balmana M, Campos D, et al. Glycosylation in the era of cancer-targeted therapy: where are we heading? *Cancer Cell*. 2019 Jul;36(1):6–16. doi: 10.1016/j.ccell.2019.06.006
- ++ A review addressing how advances in cancer-targeted therapies are increasingly focusing on glycan structures as potential biomarkers and therapeutic targets.
5. Zachara NE, Akimoto Y, Boyce M, et al. The O-GlcNAc Modification. In: Varki A, Cummings RD, Esko JD, et al. editors. *Essentials of glycobiology*. 4th ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2022. p. 251–264.
6. Reilly C, Stewart TJ, Renfrow MB, et al. Glycosylation in health and disease. *Nat Rev Nephrol*. 2019 Jun;15(6):346–366. doi: 10.1038/s41581-019-0129-4

7. Freeze HH, Boyce M, Zachara NE, et al. Glycosylation Precursors. In: Varki A, Cummings RD, Esko JD editors. *Essentials of glycobiology*. 4th ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2022. p. 53–66.
- **This book chapter explains in detail the biosynthetic and metabolic pathways involved in the synthesis of sugar nucleotide donors.**
8. Ashkani J, Naidoo KJ. Glycosyltransferase gene expression profiles classify cancer types and propose prognostic subtypes. *Sci Rep*. 2016 May;6(1):26451.
9. Kumamoto K, Goto Y, Sekikawa K, et al. Increased expression of UDP-galactose transporter messenger RNA in human colon cancer tissues and its implication in synthesis of Thomsen-Friedenreich antigen and sialyl Lewis A/X determinants. *Cancer Res*. 2001 Jun;61(11):4620–4627.
10. Cheng H, Wang S, Gao D, et al. Nucleotide sugar transporter SLC35A2 is involved in promoting hepatocellular carcinoma metastasis by regulating cellular glycosylation. *Cell Oncol (Dordr)*. 2023 Apr;46(2):283–297. doi: 10.1007/s13402-022-00749-7
11. Ju T, Lanneau GS, Gautam T, et al. Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. *Cancer Res*. 2008 Mar;68(6):1636–1646. doi: 10.1158/0008-5472.CAN-07-2345
12. Stowell SR, Ju T, Cummings RD. Protein glycosylation in cancer. *Annu Rev Pathol*. 2015 Jan;10(1):473–510.
13. Harada A, Kunii M, Kurokawa K, et al. Dynamic movement of the Golgi unit and its glycosylation enzyme zones. *Nat Commun*. 2024 Mar;15(1):4514. doi: 10.1038/s41467-024-48901-1
14. Costa AF, Campos D, Reis CA, et al. Targeting glycosylation: a new road for cancer drug discovery. *Trends Cancer*. 2020 Sep;6(9):757–766. doi: 10.1016/j.trecan.2020.04.002
- **This opinion discusses how targeting glycosylation presents a promising new approach for cancer drug discovery and therapeutic development.**
15. Morla S, Ravikumar O, O'Hara C, et al. Designing synthetic, sulfated glycosaminoglycan mimetics that are orally bioavailable and exhibiting in vivo anticancer activity. *J Med Chem*. 2023 Jan;66(2):1321–1338. doi: 10.1021/acs.jmedchem.2c01511
16. Magdalenio JSL, Grewal RK, Medina-Franco JL, et al. Toward alpha-1,3/4 fucosyltransferases targeted drug discovery: in silico uncovering of promising natural inhibitors of fucosyltransferase 6. *J Cell Biochem*. 2023 Aug;124(8):1173–1185. doi: 10.1002/jcb.30440
17. Martin KC, Tricomi J, Corzana F, et al. Fucosyltransferase-specific inhibition via next generation of fucose mimetics. *Chem Commun (Camb)*. 2021 Jan;57(9):1145–1148. doi: 10.1039/D0CC04847J
18. Debelá DT, Muzazu SG, Heraro KD, et al. New approaches and procedures for cancer treatment: current perspectives. *SAGE Open Med*. 2021 Aug;9:20503121211034366.
19. Zhong L, Li Y, Xiong L, et al. Small molecules in targeted cancer therapy: advances, challenges, and future perspectives. *Signal Transduct Target Ther*. 2021 May;6(1):201. doi: 10.1038/s41392-021-00572-w
20. Kumar M, Jalota A, Sahu SK, et al. Therapeutic antibodies for the prevention and treatment of cancer. *J Biomed Sci*. 2024 Jan;31(1):6. doi: 10.1186/s12929-024-00996-w
21. Sorensen AL, Reis CA, Tarp MA, et al. Chemoenzymatically synthesized multimeric Tn/Stn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. *Glycobiology*. 2006 Feb;16(2):96–107. doi: 10.1093/glycob/cwj044
22. Julien S, Picco G, Sewell R, et al. Sialyl-Tn vaccine induces antibody-mediated tumour protection in a relevant murine model. *Br J Cancer*. 2009 Jun;100(11):1746–1754. doi: 10.1038/sj.bjc.6605083
23. Rvalland G, Loveland B, Mitchell P. Update on Mucin-1 immunotherapy in cancer: a clinical perspective. *Expert Opin Biol Ther*. 2015 Oct;15(12):1773–1787.
24. Berois N, Pittini A, Osinaga E. Targeting tumor glycans for cancer therapy: successes, limitations, and perspectives. *Cancers (Basel)*. 2022 Jan 27;14(3):647.
25. Freitas R, Peixoto A, Ferreira E, et al. Immunomodulatory glycomedicine: introducing next generation cancer glycovaccines. *Biotechnol Adv*. 2023 Jul-Aug;65:108144.
26. Aasted MKM, Groen AC, Keane JT, et al. Targeting solid cancers with a cancer-specific monoclonal antibody to surface expressed aberrantly O-glycosylated proteins. *Mol Cancer Ther*. 2023 Oct;22(10):1204–1214. doi: 10.1158/1535-7163.MCT-23-0221
27. Xiao H, Woods EC, Vukojicic P, et al. Precision glycoalkyl editing as a strategy for cancer immunotherapy. *Proc Natl Acad Sci USA*. 2016 Sep;113(37):10304–10309. doi: 10.1073/pnas.1608069113
28. Sawada M, Moriya S, Saito S, et al. Reduced sialidase expression in highly metastatic variants of mouse colon adenocarcinoma 26 and retardation of their metastatic ability by sialidase overexpression. *Int J Cancer*. 2002 Jan;97(2):180–185. doi: 10.1002/ijc.1598
29. Uemura T, Shiozaki K, Yamaguchi K, et al. Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4. *Oncogene*. 2009 Mar;28(9):1218–1229. doi: 10.1038/onc.2008.471
30. Costa AF, Senra E, Campos D, et al. Monensin as potential drug for treatment of SLeX-positive tumors. *medRxiv*. 2024:24304048. Mar;2024.03.11.24304048.
31. Sorensen DM, Bull C, Madsen TD, et al. Identification of global inhibitors of cellular glycosylation. *Nat Commun*. 2023 Feb;14(1):948. doi: 10.1038/s41467-023-36598-7
32. Song L, Linstedt AD. Inhibitor of ppGalNAc-T3-mediated O-glycosylation blocks cancer cell invasiveness and lowers FGF23 levels. *Elife*. 2017 Mar;6:e24051.
33. Rillahan CD, Antonopoulos A, Lefort CT, et al. Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. *Nat Chem Biol*. 2012 Jul;8(7):661–668. doi: 10.1038/nchembio.999
- **This paper describes for the first time the capacity of peracetylated 3F_{ax}-Neu5Ac and 2F-Fuc as global metabolic inhibitors of sialyl- and fucosyltransferases, respectively.**
34. Li B, Kawatkar SP, George S, et al. Inhibition of Golgi mannosidase II with mannosatin A analogues: synthesis, biological evaluation, and structure-activity relationship studies. *ChemBiochem*. 2004 Sep;5(9):1220–1227. doi: 10.1002/cbic.200300842
35. Liu L, Prudden AR, Capicciotti CJ, et al. Streamlining the chemoenzymatic synthesis of complex N-glycans by a stop and go strategy. *Nat Chem*. 2019 Feb;11(2):161–169. doi: 10.1038/s41557-018-0188-3
36. Rossing E, Pijnenborg JFA, Boltje TJ. Chemical tools to track and perturb the expression of sialic acid and fucose monosaccharides. *Chem Commun (Camb)*. 2022 Nov;58(87):12139–12150. doi: 10.1039/D2CC04275D
- **This paper reviews several chemical tools that track and manipulate the expression of sialic acid and fucose monosaccharides, providing insights into their roles in biological processes and potential therapeutic applications.**
37. Del Solar V, Gupta R, Zhou Y, et al. Robustness in glycosylation systems: effect of modified monosaccharides, acceptor decoys and azido sugars on cellular nucleotide-sugar levels and pattern of N-linked glycosylation. *Mol Omics*. 2020 Aug;16(4):377–386. doi: 10.1039/D0MO00023J
38. Kizuka Y, Nakano M, Yamaguchi Y, et al. An alkynyl-fucose halts hepatoma cell migration and invasion by inhibiting GDP-fucose-synthesizing enzyme FX, TSTA3. *Cell Chem Biol*. 2017 Dec;24(12):1467–1478e5. doi: 10.1016/j.chembiol.2017.08.023
39. Zandberg WF, Kumarasamy J, Pinto BM, et al. Metabolic inhibition of sialyl-Lewis X biosynthesis by 5-thiofucose remodels the cell surface and impairs selectin-mediated cell adhesion. *J Biol Chem*. 2012 Nov;287(47):40021–40030. doi: 10.1074/jbc.M112.403568
40. Shabir G, Saeed A, Zahid W, et al. Chemistry and pharmacology of fluorinated drugs approved by the FDA (2016–2022). *Pharmaceuticals (Basel)*. 2023 Aug 15;16(8):1162.
41. Hevey R. Bioisosteres of carbohydrate functional groups in glycomimetic design. *Biomimetics (Basel)*. 2019 Jul;4(3): doi: 10.3390/biomimetics4030053
42. Coenen HH, Elsinga PH, Iwata R, et al. Fluorine-18 radiopharmaceuticals beyond [18F]FDG for use in oncology and neurosciences. *Nucl Med Biol*. 2010 Oct;37(7):727–740. doi: 10.1016/j.nucmedbio.2010.04.185

43. Sharma M, Bernacki RJ, Paul B, et al. Fluorinated carbohydrates as potential plasma membrane modifiers. Synthesis of 4- and 6-fluoro derivatives of 2-acetamido-2-deoxy-D-hexopyranoses. *Carbohydr Res.* 1990 May;198(2):205–221. doi: [10.1016/0008-6215\(90\)84293-4](#)
44. Woynarowska B, Dimitroff CJ, Sharma M, et al. Inhibition of human HT-29 colon carcinoma cell adhesion by a 4-fluoro-glucosamine analogue. *Glycoconj J.* 1996 Aug;13(4):663–674. doi: [10.1007/BF00731455](#)
45. Orozco-Moreno M, Visser EA, Hodgson K, et al. Targeting aberrant sialylation and fucosylation in prostate cancer cells using potent metabolic inhibitors. *Glycobiology.* 2023 Dec;33(12):1155–1171. doi: [10.1093/glycob/cwad085](#)
- **In this paper, the authors compare the ability of several F-MAs to target aberrant sialylation and fucosylation in prostate cancer cells.**
46. Gloster TM, Zandberg WF, Heinonen JE, et al. Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. *Nat Chem Biol.* 2011 Mar;7(3):174–181. doi: [10.1038/nchembio.520](#)
47. Woynarowska B, Skrinicosky DM, Haag A, et al. Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. *J Biol Chem.* 1994 Sep;269(36):22797–22803. doi: [10.1016/S0021-9258\(17\)31715-5](#)
48. Barthel SR, Antonopoulos A, Cedeno-Laurent F, et al. Peracetylated 4-fluoro-glucosamine reduces the content and repertoire of N- and O-glycans without direct incorporation. *J Biol Chem.* 2011 Jun;286(24):21717–21731. doi: [10.1074/jbc.M110.194597](#)
49. Dimitroff CJ, Kupper TS, Sackstein R. Prevention of leukocyte migration to inflamed skin with a novel fluorosugar modifier of cutaneous lymphocyte-associated antigen. *J Clin Invest.* 2003 Oct;112(7):1008–1018.
50. Cedeno-Laurent F, Opperman MJ, Barthel SR, et al. Metabolic inhibition of galectin-1-binding carbohydrates accentuates antitumor immunity. *J Invest Dermatol.* 2012 Feb;132(2):410–420. doi: [10.1038/jid.2011.335](#)
51. Dimitroff CJ. Leveraging fluorinated glucosamine action to boost antitumor immunity. *Curr Opin Immunol.* 2013 Apr;25(2):206–213.
52. van Wijk XM, Lawrence R, Thijssen VL, et al. A common sugar-nucleotide-mediated mechanism of inhibition of (glycosaminoglycan) biosynthesis, as evidenced by 6F-GalNAc (Ac3). *FASEB J.* 2015 Jul;29(7):2993–3002. doi: [10.1096/fj.14-264226](#)
53. Marathe DD, Buffone A Jr, Chandrasekaran EV, et al. Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins. *Blood.* 2010 Feb;115(6):1303–1312. doi: [10.1182/blood-2009-07-231480](#)
54. Jackson EG, Cutolo G, Yang B, et al. 4-Deoxy-4-fluoro-GalNAz (4FGalNAz) is a metabolic chemical reporter of O-GlcNAc modifications, highlighting the notable substrate flexibility of O-GlcNAc Transferase. *ACS Chem Biol.* 2022 Jan;17(1):159–170. doi: [10.1021/acscchembio.1c00818](#)
55. Hornik S, Cervenková Stastná L, Curinova P, et al. Synthesis and in vitro cytotoxicity of acetylated 3-fluoro, 4-fluoro and 3,4-difluoro analogs of D-glucosamine and D-galactosamine. *Beilstein J Org Chem.* 2016 Apr;12:750–759.
56. Hamala V, Ondrášková K, Štaštná L, et al. Improving the anticancer activity of fluorinated glucosamine and galactosamine analogs by attachment of a ferrocene or ruthenium tetrazene motif. *Appl Organomet Chem.* 2024 February;38(5):e7399. doi: [10.1002/aoc.7399](#)
57. Bull C, Boltje TJ, Wassink M, et al. Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. *Mol Cancer Ther.* 2013 Oct;12(10):1935–1946. doi: [10.1158/1535-7163.MCT-13-0279](#)
58. Tvaroska I, Selvaraj C, Koca J. Selectins-the two Dr. Jekyll and Mr. Hyde faces of adhesion molecules-A review. *Molecules.* 2020 Jun;25(12). doi: [10.3390/molecules25122835](#)
59. McEver RP. Selectin-carbohydrate interactions during inflammation and metastasis. *Glycoconj J.* 1997 Aug;14(5):585–591.
60. Zarbock A, Ley K, McEver RP, et al. Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow. *Blood.* 2011 Dec;118(26):6743–6751. doi: [10.1182/blood-2011-07-343566](#)
61. Trinchera M, Aronica A, Dall'Olio F. Selectin ligands sialyl-lewis x and sialyl-lewis x in gastrointestinal cancers. *Biology (Basel).* 2017 Feb;6(1). doi: [10.3390/biology6010016](#)
62. Miro L, Lopez J, Guerrero PE, et al. Sialyltransferase Inhibitor Ac(5) 3F(ax)Neu5Ac reverts the malignant phenotype of pancreatic cancer cells, and reduces tumor volume and favors T-cell infiltrates in mice. *Cancers (Basel).* 2022 Dec;14(24). doi: [10.3390/cancers14246133](#)
63. Bull C, Boltje TJ, Balneger N, et al. Sialic acid blockade suppresses tumor growth by enhancing T-cell-mediated tumor immunity. *Cancer Res.* 2018 Jul;78(13):3574–3588. doi: [10.1158/0008-5472.CAN-17-3376](#)
64. Hodgson K, Orozco-Moreno M, Goode EA, et al. Sialic acid blockade inhibits the metastatic spread of prostate cancer to bone. *EBioMedicine.* 2024 Jun;104:105163.
65. Macauley MS, Arlian BM, Rillahan CD, et al. Systemic blockade of sialylation in mice with a global inhibitor of sialyltransferases. *J Biol Chem.* 2014 Dec;289(51):35149–35158. doi: [10.1074/jbc.M114.606517](#)
66. Bull C, Boltje TJ, van Dinther EA, et al. Targeted delivery of a sialic acid-blocking glycomimetic to cancer cells inhibits metastatic spread. *ACS Nano.* 2015 Jan;9(1):733–745. doi: [10.1021/nn5061964](#)
- **In this paper, the authors develop a targeted delivery system to safely deliver 3F_{ax}-Neu5Ac to cancer cells.**
67. Moons SJ, Hornikx D, Aasted MKM, et al. UV light-induced spatial loss of sialic acid capping using a photoactivatable sialyltransferase inhibitor. *RSC Chem Biol.* 2023 Jul;4(7):506–511. doi: [10.1039/D3CB00006K](#)
68. Heise T, Pijnenborg JFA, Bull C, et al. Potent metabolic sialylation inhibitors based on C-5-modified fluorinated sialic acids. *J Med Chem.* 2019 Jan;62(2):1014–1021. doi: [10.1021/acs.jmedchem.8b01757](#)
- **This paper shows that modifications of 3F_{ax}-Neu5Ac with a carbamate residue into the C5 position lead to a higher effective inhibitor capacity.**
69. Murray BW, Wittmann V, Burkart MD, et al. Mechanism of human alpha-1,3-fucosyltransferase V: glycosidic cleavage occurs prior to nucleophilic attack. *Biochemistry.* 1997 Jan;36(4):823–831. doi: [10.1021/bi962284z](#)
70. Okeley NM, Alley SC, Anderson ME, et al. Development of orally active inhibitors of protein and cellular fucosylation. *Proc Natl Acad Sci USA.* 2013 Apr;110(14):5404–5409. doi: [10.1073/pnas.1222263110](#)
71. Zhou Y, Fukuda T, Hang Q, et al. Inhibition of fucosylation by 2-fluorofucose suppresses human liver cancer HepG2 cell proliferation and migration as well as tumor formation. *Sci Rep.* 2017 Sep;7(1):11563. doi: [10.1038/s41598-017-11911-9](#)
72. Carrascal MA, Silva M, Ramalho JS, et al. Inhibition of fucosylation in human invasive ductal carcinoma reduces E-selectin ligand expression, cell proliferation, and ERK1/2 and p38 MAPK activation. *Mol Oncol.* 2018 May;12(5):579–593. doi: [10.1002/1878-0261.12163](#)
73. Do KT, Chow LQM, Reckamp K, et al. First-in-human, first-in-class, phase I trial of the fucosylation inhibitor SGN-2FF in patients with advanced solid tumors. *Oncologist.* 2021 Nov;26(11):925–e1918. doi: [10.1002/onco.13911](#)
- **The first time an F-MAs (2F-Fuc) reaches phase I of a clinical trial.**
74. Pijnenborg JFA, Visser EA, Noga M, et al. Cellular fucosylation inhibitors based on fluorinated fucose-1-phosphates*. *Chemistry.* 2021 Feb;27(12):4022–4027. doi: [10.1002/chem.202005359](#)
- **This paper shows that the establishment of a 2F-Fuc phosphate derivative increase the potency of 2F-Fuc.**
75. Dai Y, Hartke R, Li C, et al. Synthetic fluorinated l-fucose analogs inhibit proliferation of cancer cells and primary endothelial cells. *ACS Chem Biol.* 2020 Oct;15(10):2662–2672. doi: [10.1021/acscchembio.0c00228](#)
76. Allen JG, Mujacic M, Frohn MJ, et al. Facile modulation of antibody fucosylation with small molecule fucostatin inhibitors and cocrystal structure with GDP-mannose 4,6-dehydratase. *ACS Chem Biol.* 2016 Oct;11(10):2734–2743. doi: [10.1021/acscchembio.6b00460](#)

- 198

Appendix II

Scientific Outputs

Scientific Outputs

Journal Articles

- **Costa AF**, Senra E, Faria-Ramos I, Teixeira A, Morais J, Pacheco M, Reis CA, Gomes C. ST3GalIV drives SLeX biosynthesis in gastrointestinal cancer cells and associates with cancer cell motility. **Glycoconj J**. 2023 Aug;40(4):421-433.
- **Costa AF**, Senra E, Campos A, Faria-Ramos I, Santos-Ferreira L, Lamas S, Gomes J, Pinto F, Teixeira A, Abrantes R, Duarte HO, Pacheco M, Pinto MT, Maia A, Pombinho A, Barros R, Fernandes V, Casanova-Gonçalves F, Sousa F, Barbosa J, Pereira L, Carneiro F, Reis CA, Gomes C. Monensin as potential drug for treatment of SLeX-positive tumors. **medRxiv**. 2024 Mar:2024.03.11.24304048.
- **Costa AF**, Teixeira, A, Reis. CA, Gomes, C. Novel anticancer drug discovery efforts targeting glycosylation: the emergence of fluorinated monosaccharides analogs, **Expert Opin Drug Discov**. 2025 Jan;20(2):1-11
- **Costa AF**, Hamala, V., Senra E, Teixeira A, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. 4F-GalNAc is a general inhibitor of cancer-associated O-glycans in gastrointestinal tumors. **Manuscript under preparation**
- Santos-Ferreira L, Duarte HO, Moia E, **Costa AF**, Martins A, Faria-Ramos I, Matos R, Barros R, Silva M, Pedrosa S, Batista DA, Gomes G, Borén T, Barbosa J, Sousa F, Casanova-Gonçalves F, Magalhães, A., Gomes, C, Santos-Sousa H, Bartfeld S, Carneiro F, Reis CA, Pinto F. Patient-derived organoids as an ex vivo model to study gastric glycosylation dynamics. **bioRxiv**. 2025 Fev:2025.02.21.639100
- Abrantes R, Forcados C, Warren D, Ferreira L, Senra E, **Costa AF**, Krpina K, Henrique R, Liberg AM, Bjørge L, Pinto F, Gomes C, Inderberg EM, Reis CA, Wälchli S. Restricted Sialyl-Tn chimeric antigen receptor is an effective pan- carcinoma immunotherapeutic approach. **Manuscript under revision**

Patents

- Provisional patent application: **Costa AF**, Campos D, Maia A, Pombinho A, Reis C A, Gomes C. Monensin for use in the treatment of gastrointestinal cancer through inhibition of SLeX expression and its associated malignant properties

Oral Communications

- **3º ENJIO (3º Encontro Nacional de Jovens Investigadores em Oncologia)**, Porto, Portugal, September 2021: **Costa AF**, Reis CA., Karban J, Gomes C. Análogos fluorados do monossacarídeo GalNAc como potenciais inibidores da biossíntese do antígeno tumoral STn

- **9th MCBiology symposium**, Porto, Portugal, February 2023: **Costa AF**, Senra E, Abrantes R, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **Young Researchers on stage:** International Meeting of Young Researchers – INNOGLY & GLYCONanoPROBES: Sour Turned Sweet: Glycans Bridging Technology and Precision Medicine, Iasi, Romania, April 2023: **Costa AF**, Senra E, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated O-glycans in gastrointestinal cancers.
- **Glycobasque 6**, Bilbao, Spain, November 2023: **Costa AF**, Senra E, Abrantes R, Faria-Ramos I, Reis CA., Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated O-glycans in gastrointestinal cancers.
- **EMBO Workshop: Trafficking and Glycosylation at the Golgi apparatus**, Sorrento, Italy, April 2024: **Costa AF**, Campos A, Senra E, Faria-Ramos I, Gomes J, Lamas S, Santos-Ferreira L, Pinto F, Pinto MT, Abrantes R, Teixeira A, Maia A, Pombinho A, Barros R, Casanova-Gonçalves F, Sousa F, Barbosa J, Carneiro F, Reis CA, Gomes C. Monensin disrupts Golgi function impairing SLeX biosynthesis and its malignant features in tumors

Flash communications:

- **GLUPOR 14 (14th International Meeting of the Portuguese Carbohydrate Group)**, Lisbon, Portugal, January 2023: **Costa AF**, Senra E, Abrantes R, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **29th Porto Cancer Meeting**, Porto, Portugal, May 2023: **Costa AF**, Senra E, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **IVSPG- IV International Meeting of the Portuguese Society of Genetics**, Braga, Portugal, October 2023. **Costa AF**, Senra E, Faria-Ramos I, Teixeira A, Morais J, Pacheco M, Reis CA, Gomes C. ST3GalIV promotes the biosynthesis of SLeX in gastrointestinal cancer cells and is linked to cancer cells motility.
- **International SialoGlyco meeting**, Lille, France, June 2024: **Costa AF**, Hamala V, Senra E, Teixeira A, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. 4F-GalNAc as a novel inhibitor of cancer-associated O-glycans in gastrointestinal cancer cells.

Posters communications:

- **ICS 2022– 30th International Carbohydrate Symposium**, Brazil (Online): **Costa, AF**, Campos D, Senra E., Maia A., Pombinho A., Reis CA., Gomes C. High throughput screening of chemical compounds showed monensin as novel cancer drug by inhibiting SLeX expression and malignant properties in gastrointestinal cancer cells
- **GLUPOR 14 (14th International Meeting of the Portuguese Carbohydrate Group)**, Lisbon, Portugal, January 2023: **Costa AF**, Senra E, Abrantes R, Reis C A, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **5thPhDay**, Porto, Portugal, October 2023: **Costa AF**, Senra E, Hamala V, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **15th Jenner Glycobiology and Medicine Symposium**, Porto, Portugal, June 2023: **Costa AF**, Senra E, Hamala V, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **Carbohydrates GRC (Gordon Research Conference): Basic and Translational Glycoscience for Human Health and Diseases**, Holderness, USA, June 2023: **Costa AF**, Senra E, Hamala V, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated truncated O-glycans in gastrointestinal cancer.
- **IVSPG- IV International Meeting of the Portuguese Society of Genetics**, Braga, Portugal, October 2023. **Costa AF**, Senra E, Faria-Ramos I, Teixeira A, Morais J, Pacheco M, Reis CA, Gomes C. ST3GalIV promotes the biosynthesis of SLeX in gastrointestinal cancer cells and is linked to cancer cells motility.
- **International SialoGlyco meeting**, Lille, France, June 2024: **Costa AF**, Hamala V, Senra E, Teixeira A, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. 4F-GalNAc as a novel inhibitor of cancer-associated O-glycans in gastrointestinal cancer cells.
- **6thPhDay**, Porto, Portugal, October 2024: **Costa AF**, Hamala V, Senra E, Teixeira A, Abrantes R, Faria-Ramos I, Reis CA, Karban J, Gomes C. Fluorinated GalNAc as a novel inhibitor of cancer-associated O-glycans in gastrointestinal cancer.

Awards:

- i3S Fundraising Cancer Travel Grant by TabFund (1st edition) to attend Carbohydrates GRC (Gordon Research Conference);

- Travel Grant to attend EMBO Workshop: Trafficking and Glycosylation at the Golgi apparatus;
- Best poster presentation at the International SialoGlyco meeting.

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