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Innovative Nanoparticles for Targeted Therapy Delivery in Gastrointestinal Cancer

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INNOVATIVE NANOPARTICLES FOR TARGETED THERAPY DELIVERY IN GASTROINTESTINAL CANCER

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“It always seems impossible until it’s done.”

Nelson Mandela

ABSTRACT

Gastric cancer (GC) is the fourth leading cause of cancer-related deaths worldwide, confirming the urgent need to develop new and more effective therapeutic approaches. Nanoparticles (NPs) represent a potential approach for drug delivery in cancer, including GC, due to their controlled release, capacity to encapsulate hydrophobic drugs and targeting ability features.

Tyrosine kinase inhibitors (TKI) are small molecules known as inhibitory drugs that can block the growth and spread of tumor cells by interfering with key molecules involved in malignancy. However, their effectiveness is limited by the acquired resistance, hydrophobicity or adverse effects like toxicity. These problems can be avoided by TKI encapsulation in a nanosystem and its delivery in a controlled manner.

Foretinib is an oral multikinase inhibitor targeting MET and RON receptors tyrosine kinases (RTK) that has been used in clinical trials for several solid tumors and the studies report some problems of uptake. Regarding Lapatinib, a dual TKI (HER2 and EGFR), its clinically used in a restrictive form and is characterized by extensive albumin binding capacity and poor water solubility, which results in poor and incomplete absorption from the gastrointestinal tract. These both TKIs represent a good example of drugs that have problems that could be avoided if they were encapsulated in a nanodelivery system and delivered to a target.

To increase the specificity of these nanodelivery systems, antibodies have been identified as potential target ligands. Glycans, highly expressed by cancers cells, are attractive targets for such delivery strategy. Therefore, antibodies targeting these aberrant glycans structures might be chemically conjugated to the surface of the NPs. Particularly, sialylated glycans, such as Sialyl-Tn (STn) have been identified as abnormally expressed in several epithelial tumors, including GC, and rarely expressed in normal tissues. This characteristic makes STn a promising targeting molecule in a delivery nanosystem.

Regarding this, the three big aims of this doctoral project were: to develop NPs surface-functionalized with a specific antibody, B72.3, targeting the STn glycan; to encapsulate individually different TKIs such Lapatinib and Foretinib; and to evaluate the effectiveness of the developed nanosystem regarding its specificity and recognition capacity in multicellular tumor spheroids (MCTS) and in *in vivo* models expressing STn.

In this work, the NPs surface-functionalized with anti-STn antibody were demonstrated to be efficiently recognized by cells displaying the cancer-associated STn

antigen under static and live cell monitoring flow conditions. This uncovers the potential use of such NPs for drug delivery in cancer. Here we presented an innovative and successful methodology to live track the NPs targeting STn antigen under shear stress, simulating the physiological flow. This robust approach can be applied for in vitro drug studies, giving valuable insights for in vivo studies.

Moreover, our data indicated that NPs loaded with Foretinib and directed to STn specifically target gastric cancer MCTS expressing the STn glycan (MKN45 SimpleCell (SC)), leading to a decrease of activation of phospho RTK and reduced cell viability.

Finally, *in vivo* evaluation using MKN45 SC xenograft mice revealed that NPs functionalized with antibody anti-STn and carrying Foretinib were able to decrease tumor growth, reduce cell proliferation and tumor necrosis area. Furthermore, tumors treated with these nanodelivery system also exhibited decreased activation of the RTK MET and RON.

Overall, this thesis demonstrates the value and relevance of using a NP targeting STn antigen for drug delivery, highlighting its potential for the therapeutic application in GC and in other tumors expressing this cancer-associated glycan.

RESUMO

O cancro gástrico (CG) é a quarta principal causa de morte relacionada com cancro em todo o mundo. Desta forma, existe uma necessidade urgente de desenvolver novas abordagens terapêuticas mais eficazes. As nanopartículas (NPs) representam uma abordagem com alto potencial para entrega de fármacos em cancro, incluindo CG, devido às suas características de libertação controlada do fármaco e capacidade de direcionamento da NP.

Os inibidores de tirosina quinase (ITQ) são pequenas moléculas capazes de inibir e bloquear o crescimento e a disseminação de células tumorais, interferindo com os recetores de tirosina quinase (RTQ), que estão envolvidas na agressividade da doença. No entanto, a sua eficácia é limitada devido a mecanismos de resistência celular e a efeitos adversos associados como a toxicidade. De modo a ultrapassar estas limitações os nanossistemas, que permitem a entrega controlada de fármacos (ITQ) e a sua encapsulação, representam excelentes alternativas para melhorar estas terapias.

O Foretinib é um inibidor oral de multiquinases que tem como alvo os RTQ, MET e RON. Este fármaco tem sido usado em ensaios clínicos para vários tumores sólidos e os estudos reportam alguns problemas na sua eficiência. Em relação ao Lapatinib, um ITQ duplo (HER2 e EGFR), o seu uso clínico é feito de forma restritiva e é caracterizado por extensa capacidade de ligação à albumina, baixa biodisponibilidade oral e baixa solubilidade em água, o que resulta numa absorção má e incompleta pelo trato gastrointestinal. Os ITQs acima mencionados são, na atualidade, excelentes exemplos de fármacos que apresentam limitações e cuja atividade e biodisponibilidade podem ser melhorados. Ao serem encapsulados em nanossistemas direcionados para moléculas alvo evita-se efeitos adversos que lhe estão associados.

De modo a melhorar a especificidade das NPs, os anticorpos representam excelentes ligandos que podem reconhecer os glicanos expressos de forma aberrante por células tumorais. Assim, a presença de glicanos sobreexpressos por células tumorais são alvos atraentes para esta estratégia de direcionamento de fármacos. Os glicanos sialilados, como o Sialil-Tn (STn), são expressos de forma anormal em vários tumores epiteliais, incluindo no CG, sendo o STn raramente expresso em tecidos normais, constituindo desta forma, um potencial alvo para um nanossistema.

Os três grandes objetivos deste projeto de doutoramento foram: o desenvolver de NPs funcionalizadas na superfície com um anticorpo específico, B72.3, direcionado para o glicano STn; encapsular individualmente diferentes ITQs como Lapatinib e Foretinib; e avaliar a eficácia deste nanossistema relativamente à sua especificidade e

capacidade de reconhecimento em esferoides tumorais e modelos *in vivo* que expressam STn.

Nesta tese, os resultados obtidos mostraram que as NPs funcionalizadas à superfície com o anticorpo anti-STn são reconhecidas eficientemente e especificamente por células tumorais que expressam STn em condições de cultura celular estática e com fluxo. Isto revela o potencial destas NPs para serem usadas na entrega de fármacos em cancro. Aqui apresentamos uma metodologia inovadora e bem-sucedida para rastrear e transmitir ao vivo as NPs direcionadas ao STn sob efeito de um estímulo, simulando o fluxo fisiológico. Esta abordagem robusta pode ser aplicada futuramente em estudos de fármacos *in vitro*, fornecendo informações valiosas para estudos posteriores.

Posteriormente, resultados obtidos indicaram que NPs com Foretinib encapsulado e direcionadas para o STn, ligaram-se especificamente a esferoides de CG que expressam o glicano STn (MKN45 SimpleCell (SC)), levando à diminuição da ativação da fosforilação dos RTQ e redução da sua viabilidade celular.

Por fim, a avaliação *in vivo* realizada com ratinhos xenografados com a linha celular MKN45 SC revelou que NPs com Foretinib encapsulado e funcionalizadas com anticorpo anti-STn, foram capazes de diminuir o crescimento tumoral, reduzir a proliferação celular e a área de necrose tumoral. Além disso, os tumores tratados com esse sistema de nanoentrega também apresentaram inativação de fosforilação de MET e RON.

Este trabalho demonstra assim a importância e a relevância do uso de NPs direcionadas ao glicano STn como sistemas de entrega de fármacos, destacando o seu potencial para aplicação terapêutica em CG e noutros cancros que expressem este glicano.

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ABBREVIATIONS

AuNPs - Gold-based NPs

APC - Antigen-presenting cells

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

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

CA 72-4 - Carbohydrate antigen 72-4

CDH1 - Cadherin 1 gene

CEA - Carcinoembryonic antigen

CIN - Chromosomally unstable tumors

COOH – Carboxylic group

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

EBV - *Epstein–Barr* virus

EBV+ - positive for Epstein-Barr virus

ECM - Extracellular matrix

EDC - 1-ethyl-3(3-dimethylaminopropyl) carbodiimide

EGFR - Epidermal growth factor receptor 1

EMA - European Medicine Agency

EMT - Epithelial-to-mesenchymal transition

EPR - Enhanced permeability and retention

ER - Endoplasmic reticulum

FDA - US Food and Drug Administration

FGFR2 - Fibroblast growth factor receptor 2

FRT – Foretinib

GAG – Glycosaminoglycan

GALNT - GalNAc-transferase

GC – Gastric Cancer

GlcNAc - N-acetylglucosamine

GSL – Glycosphingolipid

HA - Hyaluronic acid

HDGC - Hereditary diffuse gastric cancer

HER2 - Human epidermal growth factor receptor 2

Le^x - Lewis x

LPT – Lapatinib

MES - 2-(N-morpholino) ethanesulfonic acid

MSI - Microsatellite unstable tumors

Neu5Ac - N-acetylneuraminic acid

NHS - N-hydroxysuccinimide

NPs – Nanoparticles

PLGA - Poly(lactic-co-glycolic acid)

PEG - Polyethylene glycol

RTK - Receptor tyrosine kinase

SLe^a - Sialyl Lewis A

SLe^x - Sialyl Lewis x

STn - Sialyl-Tn

T - Thomsen–Friedenreich antigen

TCGA - The Cancer Genome Atlas

TKI - Tyrosine kinase inhibitor

VEGFR - Vascular endothelial growth factor receptor

WHO - World Health Organization

CHAPTER 1

General Introduction

Aims

This chapter contains parts of the following literature review:

Appendix I: Diniz F, Coelho P, Duarte HO, Sarmento B, Reis CA, Gomes J. Glycans as Targets for Drug Delivery in Cancer. *Cancers*. 2022; 14(4):911. <https://doi.org/10.3390/cancers14040911>

GENERAL INTRODUCTION

1. Gastric Cancer

Nowadays, cancer is considered in the top of the most contributors to premature mortality in majority countries worldwide. Along of this century, is expected that cancer will become the prominent cause of premature death of the world and principal incident disease (1). The reasons behind this scenario can reflect both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer (2).

Gastric cancer (GC) represents a major health problem in many countries, essentially in Asia and Europe (2, 3). This disease constitutes an extremely aggressive health problem resulting in high mortality rates (4).

1.1 Epidemiology and Risk Factors

According with GLOBOCAN, GC constitutes the fifth most incident of cancer disease. In 2020 the incidence worldwide was around 1 million (1 089 103) of new cases of GC, which represents 5.6% of new cases identified (**Figure 1A**). From this 1 million diagnosed cases results 768 793 (7.7%) deaths, that confers the GC the fourth most mortal cancer disease (2) (**Figure 1B**).

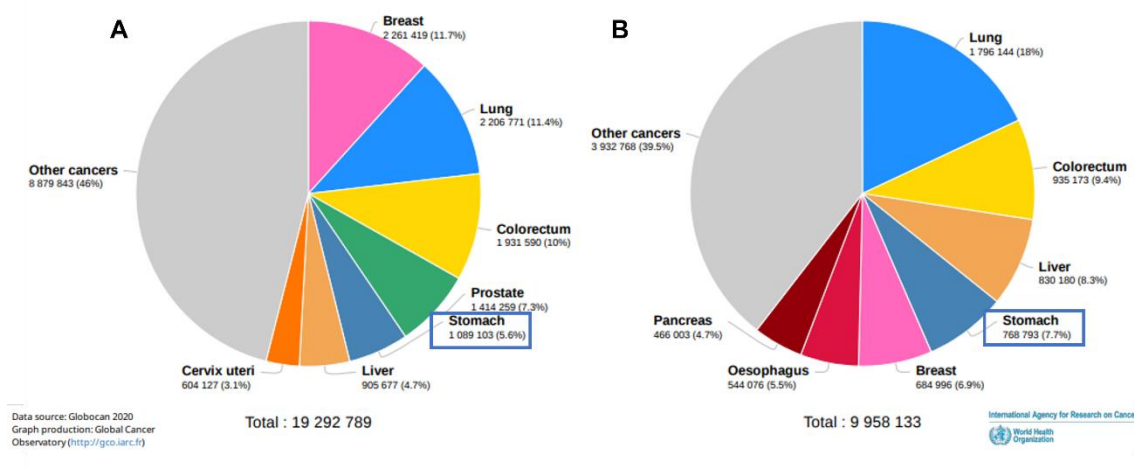


Figure 1: Estimated number incidence of cancer (**A**) and respective mortality (**B**) in the world in 2020 for both genders and all ages, according with data source GLOBOCAN (Adapted from(2)).

Despite these high values, the distribution of the disease is heterogenous displaying a substantial global variation in incidence. The highest rates are observed in Eastern Asia, Central and Eastern Europe and South America (2, 4). More precisely, more than 50% of the new cases occur in developing countries. Moreover, is also described that the incidence in men is twice as high as in women (4). GC is a multifactorial disease, since

many factors can influence its development, both environmental and genetic, and this could explain this heterogeneity in world incidence (5).

Several factors have been associated with the increased risk of developing GC, like family history, diet, alcohol consumption, smoking, *Epstein–Barr* virus (EBV) and, one of the primary causes, *Helicobacter pylori* infections (5, 6) (**Figure 2**).

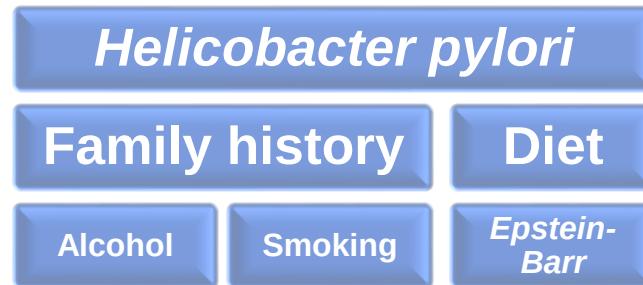


Figure 2: Risk Factors associated with gastric cancer disease: *Helicobacter pylori* infection, family history, diet, alcohol consumption, smoking and *Epstein-Barr* virus infection.

H. pylori infection is considered a common cause of gastrointestinal diseases, however only a few part infected patients developed gastric severe complications: 10-15% peptic ulcer and 1-3% GC (6). The infection with *H. pylori* is mainly acquired in early infancy and remains present for life if not detected and treated with antibiotics (7). Chronic gastritis caused by *H. pylori* infection is associated to the development of the carcinogenic process, thus *H. pylori* eradication may prevent GC development (4, 5, 7).

A family history of GC is also one of the most crucial risk factors. GCs are mostly sporadic, however, around 10% display a familial aggregation and a familial genetic predisposition is identified in around 3% of cases (4). The most recognized familial GC is hereditary diffuse gastric cancer (HDGC), which is caused by cadherin 1 gene (CDH1) alterations. The risk of GC associated to patients with a family history, compared to individuals without such a history, is around three-fold higher(5).

1.2 Pathology Classification and Clinical Outcome

There are multiple GC histopathological classification systems, however the most commonly used is the Lauren classification, which distinguishes two major subtypes of GC: intestinal (with intercellular junctions) and diffuse (without intercellular junctions) subtypes (5-7). The most common is the intestinal subtype, the second is diffuse and ending with the indeterminate subtype, that appeared latter to characterize infrequent histology (5).

Intestinal carcinoma is distinguished by visible glands and cohesion between tumor cells, in the other hand, the diffuse subtype included poorly cohesive cells, diffusely infiltrating

the gastric wall with almost no gland formation (5, 7). There is evidence that intestinal metaplasia of the gastric mucosa and the occurrence of *H. pylori* infection can be linked to intestinal subtype emergence. Some studies also demonstrated that the incidence of the diffuse GC subtype is higher between females and younger patients, and that this subtype of GC is associated to normal gastric mucosa (5). The ratio of both subtypes is heterogeneous between countries and continents. In Europe, intestinal subtype is currently more prevalent (6).

Other classifications, more complex, are proposed by the World Health Organization (WHO) and the Japanese Endoscopic Society (4). The WHO classification is distinguished as the most detailed among all classifications systems. In addition to stomach adenocarcinomas (that represent 90% of GC) (4), also describes other types of GC with lower attendance, which are similar to the indeterminate subtype according to the Lauren classification system (5).

GC is often asymptomatic in the early stages delaying disease diagnosis. Due to the frequently late disease diagnosis, the majority of GC patients end up with a worrying clinical outcome. Complete or partial gastric surgical resection remains the only available curative therapeutic solution, that confers a 5-year-survival rate of 50-70% to early diagnosed patients. In the other hand, in patients diagnosed with locally advanced or metastatic unresectable disease, 5-year-survival rates decrease to a tragic 4-10% (8, 9). The aggressivity of GC disease and consequently poor outcomes, even in the setting of resectable disease, mean that the concept of survivorship is only now beginning to evolve (4).

1.3 Molecular Classification

The Cancer Genome Atlas (TCGA) research network has identified four tumor subgroups in gastric carcinomas: positive for Epstein-Barr virus (EBV+) (9%), microsatellite unstable tumors (MSI) (22%), genomically stable tumors (20%), and chromosomally unstable tumors (CIN) (50%) (10).

Each subgroup is characterized by selected molecular abnormalities, with some convergences (4). Classification of GCs based on molecular subtypes might be used in future to determine prognosis and to adapt to the best treatment option. The molecular characteristics of CIN and MSI tumors are the best understood of the among subgroups (10).

Succinctly, EBV+ tumors are featured by PIK3CA mutations and PD-L1/2 amplifications. Tumors with MSI have high mutation rates, that has a particular impact on key signaling oncogenic proteins, such as human epidermal growth factor receptor 2 (HER2) and the

epidermal growth factor receptor 1 (EGFR). For the genomically stable tumors, mutations of genes codifying for E-cadherin (CDH1) and the RHO-family GTPase-activating proteins can be observed. Finally, tumors of the CIN subtype present marked aneuploidy, TP53 mutations and focal amplifications of Ras proteins. This subtype is enriched for copy number changes in key receptor tyrosine kinase (RTK) oncogenes such as HER2, EGFR, fibroblast growth factor receptor 2 (FGFR2) and MET (4, 11).

1.4 Serological Biomarkers

Generally, single tumor biomarkers are not enough sensitive and specific to detect GC, for this reason, the combined detection using different markers is essential. In cases of GC, serum CA 19-9, carcinoembryonic antigen (CEA) and carbohydrate antigen 72-4 (CA 72-4) are the three most used serological biomarker and are important during an early GC diagnosis and therapy monitoring (12-14).

CA 19.9 is known to detect a glycan structure commonly upregulated in GC, sialyl Lewis A (SLe^a). The utility of CA 19-9 as a diagnostic biomarker of GC is slightly controversial, as benign diseases (inflammatory process) often overexpress this glycan as well. However, it can be used in the clinical practice to monitor recurrence and response to therapy in GC patients (12, 13). The increased of both markers CA 19-9 and CA 72-4 levels are associated with a poor prognostic among GC patients (15).

CA 72.4 allows to detect high levels of sialyl-Tn (STn) in the serum of GC patients and can be used as an independent prognostic factor of tumor aggressiveness and recurrence and poor prognosis to GC patients (15, 16). Nevertheless, pre-cancerous lesions of the stomach can express STn and can cause a high false-positive rate, limiting its clinical applicability for GC diagnosis (15, 16).

Lastly, the CEA serological assay is able to detect the highly glycosylated CEA protein, also very frequently overexpressed in GC. Despite that, sensitivity limitations of the CEA serological method prevent premature stages detection of GC (17).

1.5 Personalized Therapeutic Strategies

A new trend in patients with advanced GC have been the introduction of targeted therapies by applying monoclonal antibodies or small molecule inhibitors against aberrant molecular alterations of the given patients' cancer. Currently, Trastuzumab and Ramucirumab(18) are two antibodies with use clinic in GC to target the oncogenic overexpression of HER2 and vascular endothelial growth factor receptor (VEGFR), respectively (10).

Approximately 17–20% of patients with GC are reported with overexpression of the HER2. These patients can benefit from treatment with the anti-HER2 antibody trastuzumab (19). According to the results of the ToGA trial, trastuzumab in combination with capecitabine or 5-FU and cisplatin is now the standard of care for HER2-positive GCs (20).

It has been associated that among GC cases, VEGF status and serum levels are correlated with advanced stage and poor prognosis (21). Thus, Ramucirumab has been evaluated as second line therapy after disease progression on a first line chemotherapy regimen, among cases with unresectable, advanced gastroesophageal tumors (18). Moreover, a phase III study demonstrated that Ramucirumab in addition to paclitaxel has correlated with a survival benefit and progression compared with paclitaxel alone (22).

Several other antibodies and small molecule inhibitors are currently being evaluated for GC in ongoing clinical studies in order to becoming future options for personalized treatments.

1.5 .1 Clinical Trials with Tyrosine Kinase Inhibitors

There are several receptors tyrosine kinase (RTKs) that have been described as involved in GC development. The main signatures of GC disease development included the expression of these receptors that regulate cancer cell growth, apoptosis, cell cycle regulators, invasion and proliferation. Therefore, small molecule inhibitors denominated tyrosine kinase inhibitors (TKIs) directed to these targets have been developed during the last two decades in order to inhibit some of these receptors. In general, they target the active site of the receptor and in this way prevent the phosphorylation of intracellular targets. These targets are often involved in cancer cell proliferation and angiogenesis (23).

Lapatinib

Lapatinib (LPT) is a potent reversible and selective dual inhibitor of the tyrosine kinase receptors EGFR and HER2. It exerts its action by competitive binding to the intracellular ATP-binding site of each receptor. This leads to downstream blocking of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin dependent transduction pathways, resulting in growth arrest or apoptosis of tumor cells (24) (**Figure 3**). As we mentioned previously, HER2 is overexpressed in 17%-20% of GC patients (19) and EGFR is overexpressed in 27%–64% of GC patients (25, 26), representing a generous percentage of GC patients that could be targeted and treated by LPT.

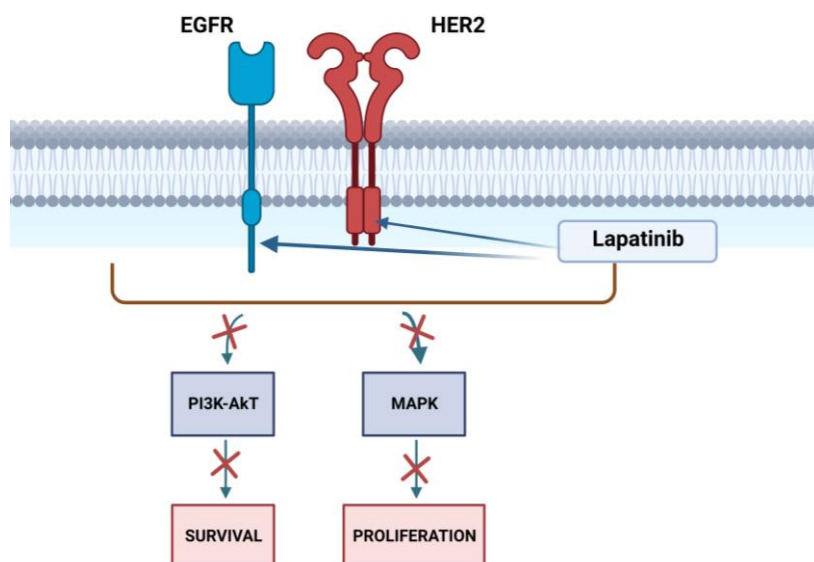


Figure 3: Mechanism of action of Lapatinib in EGFR and HER2 tyrosine kinase receptors.

Multiple studies *in vitro* demonstrated the impact of LPT treatment in GC cells(27-29). In metastatic HER+ breast cancer, LPT is it approved and can be used as a first line in addition to trastuzumab in selected patients, since this treatment is super toxic for the patient. In the second line therapy, LPT seems less effective than the antibody-dug conjugate trastuzumab emtansine (30). Nevertheless, despite being well-tolerated, LPT antitumor effect demonstrated to be insufficient, both as a single-agent or in combination with systemic chemotherapy, in significantly improving the prognosis of patients diagnosed with advanced GC (31, 32). A phase III study also demonstrated that LPT plus paclitaxel showed activity in the second line treatment of patients HER2+ diagnosed with advanced GC, however, did not significantly improve overall survival (33).

LPT is being clinically used in a restrictive form and is associated with extensive albumin binding capacity, poor oral bioavailability, and poor water solubility. This results in poor and incomplete absorption from the gastrointestinal tract. Moreover, this drug is commercialized for oral intake only(34, 35). Thus, it becomes crucial developed efficient alternatives for administration through non-oral routes.

Foretinib

Foretinib (FRT) is an oral multikinase inhibitor, which exhibits activity against MET and a number of other TKRs engaged in angiogenesis associated to neoplastic disease, such as RON, Tie-2, PDGFR- β , AXL and VEGFR (36-39). FRT is an ATP-competitive inhibitor, therefore, similar to LPT, binds with high affinity deep in the ATP pocket of tyrosine kinase domains (40) (**Figure 4**). MET is amplified in approximately 20% of the

diffuse subtype of GC and when aberrantly activated, MET is a key driver of tumor cell growth, motility, invasion, metastasis and angiogenesis (41, 42). RON is also a RTK, activated by a macrophage stimulating protein growth factor responsible for playing a role in tumorigenesis and is associated with poor clinical outcome. In GC, RON is described to be expressed in about 50% of GC tissues (43). VEGFR receptor is expressed in vascular endothelial cells and is through it that the mesenchymal tissues can promote angiogenesis, playing a critical role in tumor progression (44, 45). In early GC tissues, the expression positive rate of VEGFR is about 75%, is also associated a poor prognosis (44, 46).

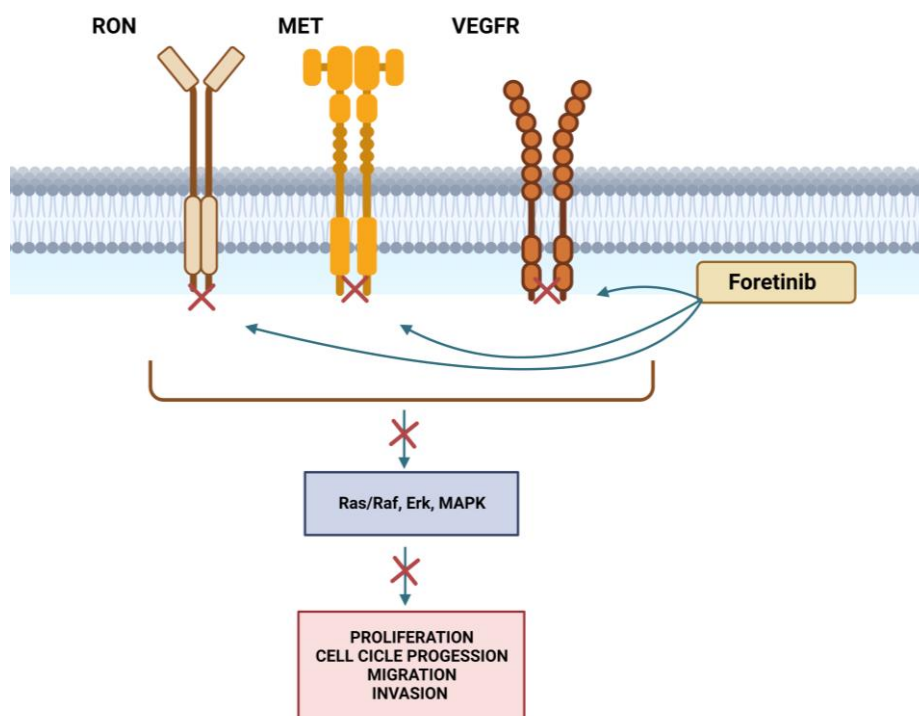


Figure 4: Mechanism of action of Foretinib in RON, MET and VEGFR tyrosine kinase receptors.

Several *in vitro* studies have demonstrated the efficiency and the potential of FRT (38, 47, 48). FRT antitumor potential has been studied in phase I/II trials in a variety of advanced solid tumors (49-52). Besides molecular evidence of target engagement and inhibition and efficiency of the drug, in a phase II clinical trial, FRT-based therapy demonstrated to be insufficient in prolonging the overall survival of unselected patients harboring metastatic GC (52). In Phase I trials, FRT inhibited activation of MET and decreased the proliferation of solid tumors in patients, however, not all GC patients carrying genomic MET amplification are sensitive to FRT (49, 53). These results associated to the lack of efficiency of this TKI lead to the discouraged further clinical investigation (ClinicalTrials.gov Identifier: NCT02016534).

2. Glycobiology in Cancer

During the last years, glycosylation has revealed to have an important role in cancer research, providing a set of targets that could be used for diagnostic application and therapeutic strategies as well (54-56). Glycosylation consists in an important and well-regulated mechanism of secondary protein processing within cells, and which is able to regulate and control several physiopathological processes. Glycans are involved in multiple processes such as, cell signaling and communication, tumor cell dissociation and invasion, cell-matrix interplay, tumor angiogenesis, metastasis development and immune modulation (54, 57, 58). The alterations in glycosylation regulate the development and progression of cancer, thus, glycans can be important biomarkers and provide specific targets to develop novel therapeutic interventions(54, 58).

2.1 Protein Glycosylation

Glycosylation is a highly regulated enzymatic process comprising the addition of an initial monosaccharide to a non-glycosyl aglycone, usually a protein or a lipid. The initial sugar is further elongated with additional monosaccharides, which are linked covalently through glycosidic bonds, forming oligosaccharides or polysaccharides. Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus, where the highly orchestrated and organized activity of both glycosyltransferases and glycosidases produces a vast repertoire of structurally complex and functionally diverse carbohydrate structure, or glycans [8] (**Figure 5**).

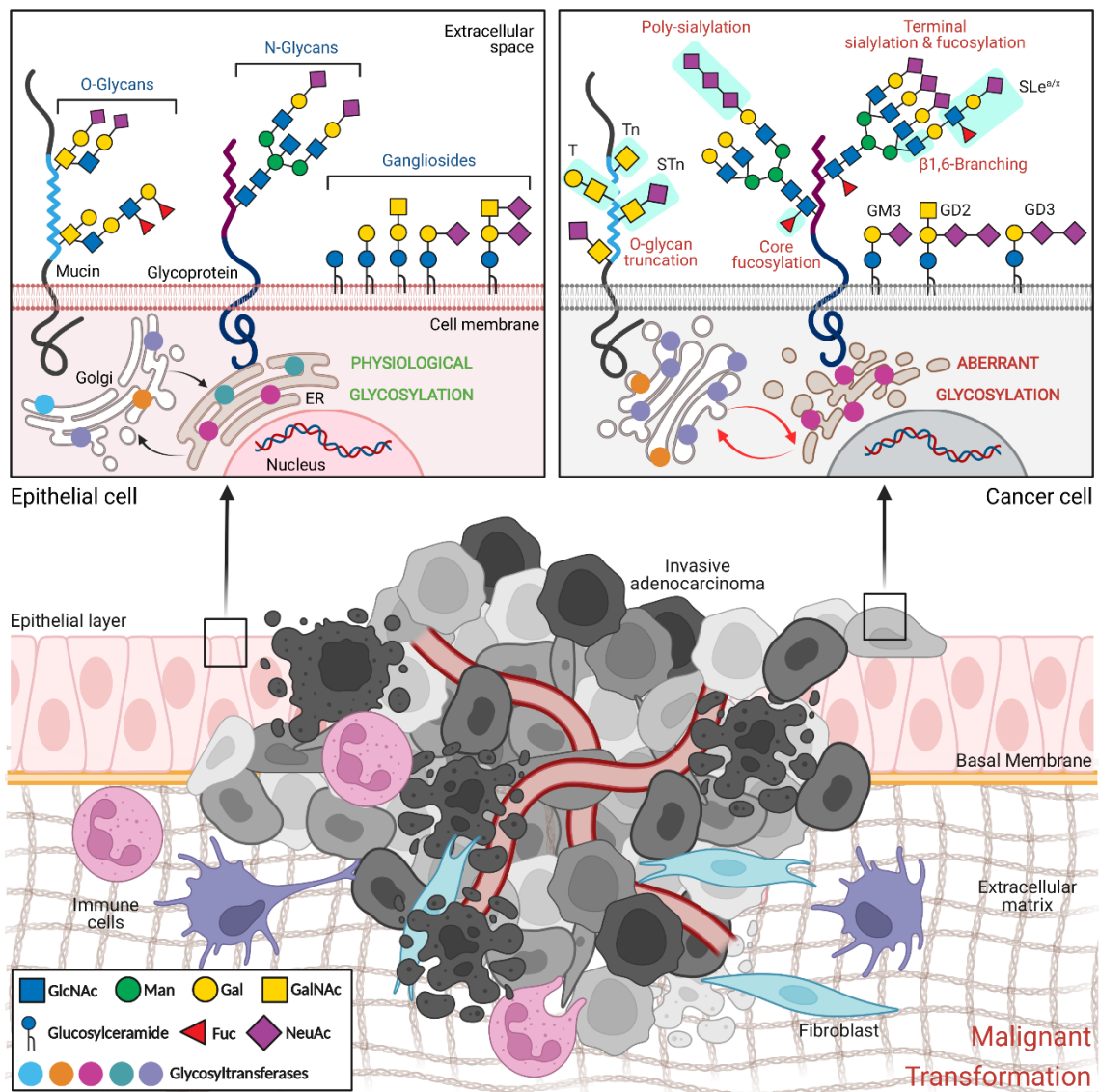


Figure 5: Glycosylation alterations during malignant transformation. Abbreviations: ER—endoplasmic reticulum; Fuc—fucose; Gal—galactose; GlcN—glucosamine; GlcA—glucuronic acid; GlcNAc—N-acetylglucosamine; GalNAc—N-acetylgalactosamine; IdoA—iduronic acid; Man—mannose; Neu5Ac—N-acetylneuraminic acid; STn—sialyl Tn; SLe^{a/x}—sialyl Lewis a/x; T—Thomsen–Friedenreich antigen; Xyl—xylose. (Extracted from Diniz et al. 2022(59))

In protein glycosylation, glycans are typically attached via glycosidic linkages to the nitrogen atom on the side chain of asparagine (N-glycans)(60) or the oxygen atom on the side chain of threonine or serine (O-glycans) (61). Other important type of glycoconjugates are glycosphingolipids (GSLs), cell-surface glycolipids formed by a hydrophobic ceramide core and a hydrophilic residue (62). On the other hand, proteoglycans are glycoconjugates with one or more glycosaminoglycan (GAG) chains, such as chondroitin sulfate, heparan sulfate and keratan sulfate, attached to a protein backbone (63).

Glycans are produced in a non-templated way and their synthesis is controlled by substrate availability, gene transcription levels, tissue-specific expression patterns of glycosyltransferases and glycosidases, enzyme location within the compartments of the secretory pathway, chaperone activity, donor substrate availability, and modifications in the tertiary conformation of acceptor peptides (54, 64).

Cell surface glycoconjugates form the glycocalyx, a dense gel-like protective barrier that shields the plasma membrane from physical stress while also shaping various aspects of cell surface dynamics. Furthermore, cell surface glycoconjugates are key regulators of various molecular and cellular processes, including intracellular trafficking, cell–cell interactions, cell–matrix adhesion, as well as molecular signaling, immune regulation, host-pathogen recognition and malignant transformation (54) (**Figure 5**).

In cancer, aberrant expression and mislocalization of glycosyltransferases and glycosidases, as well as imbalances affecting the availability of sugar nucleotide donors, lead to the emergence of aberrant glycosylation patterns (**Figure 5**). These cancer-derived glycan structures are key players in tumor biology, as they actively support to the various neoplastic characteristics of malignant cells (65). In fact, it has been extensively demonstrated that cancer-associated glycans mediate the crosstalk between cancer cells and their surrounding microenvironment, contributing for the acquisition of cancer hallmarks, such as sustained proliferative signaling, resistance to cell death, immune system evasion, angiogenesis, invasion and metastization (54) (**Figure 5**). RTKs and their downstream signaling cascades actively drive neoplastic transformation. Moreover, several studies have demonstrated that the altered glycosylation of different RTKs (such as EGFR, HER2, MET, and RON) leads to their hyperactivation, which, in turn, promotes RTK-dependent malignant growth and phenotypically aggressive tumors (66-70).

2.2 O-glycosylation and Alterations in Gastric Cancer

O-glycosylation produces mature, elongated, and branched O-glycan chains, often terminally modified with sialic acids or N-acetylneuraminic acid (Neu5Ac) through the action of sialyltransferases. A set of 20 GalNAc-transferases (encoded by GALNTs) initiates the biosynthesis of O-GalNAc glycan chains(71). This family of enzymes transfers GalNAc to Ser/Thr residues of proteins, forming the Tn antigen (GalNAc α 1-O-Ser/Thr) (**Figure 6**). Subsequently, the Tn antigen is modified by different glycosyltransferases, forming complex and mature O-glycans. Glycosyltransferase core 1 GalNAc β 1,3-galactosyltransferase 1 (C1GalT1) is responsible for adding galactose (Gal) to the Tn antigen, forming the core 1 O-glycan structure or the T antigen (Gal β 1-

3GalNAc α 1-O-Ser/Thr). Core 1 β 3-Gal-T specific molecular chaperone (COSMC), localized in the ER, is necessary for the activation of C1GalT1 in the Golgi apparatus by preventing its misfolding, aggregation and proteasomal degradation(72). Core 1 structures can be further modified by core 2 β 1,6-N-acetylglucosaminyltransferase (C2GnT), which adds N-acetylglucosamine (GlcNAc) to the T antigen, forming core 2 O-glycans. These structures can then be subsequently modified by different glycosyltransferases, including sialyl- and fucosyltransferases, forming increasingly complex structures (**Figure 6**) (61).

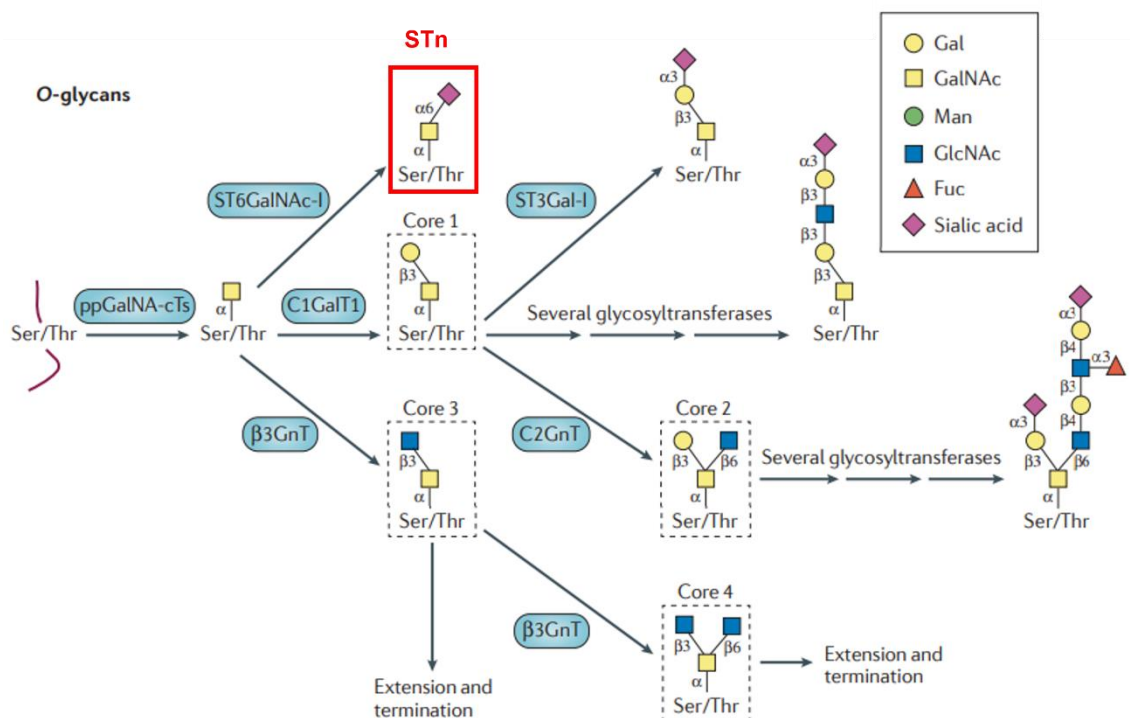


Figure 6: Representation of important O-linked glycan structures, as well as the key enzymes known to add specific sugar residues (blue boxes). Framed with a red box is the truncated O-glycan STn, that has been studied in this thesis. (Adapted from(54))

In cancer, alterations affecting the glycosylation machinery trigger the expression of immature and truncated O-glycans, such as Tn antigen, and its sialylated form, STn (NeuAc α 2-6-GalNAc α 1-O-Ser/Thr) (54). In non-transformed cells, early-acting enzymes responsible for core O-glycan biosynthesis, such as C1GalT1 and C2GnT, are localized in cis- and medial-Golgi, and late-acting enzymes, such as sialyltransferases, are localized in the trans-Golgi. In the cancer setting, however, the overexpression of sialyltransferases leads to their aberrant distribution across all the Golgi cisternae, where they will compete with early acting-enzymes and contribute to the emergence of immature forms of O-glycan chains. One example is the overexpression of the α -GalNAc

α 2,6-sialyltransferase I (ST6GalNAc1) that leads to the premature addition of sialic acid to form the STn antigen (**Figure 6**) (73, 74).

In cancer, several glycoproteins are overexpressed and aberrantly glycosylated, carrying tumor-associated glycans underpinning tumor growth and metastization(65). CD44, a transmembrane receptor for hyaluronic acid (HA) and other ECM molecules, is a well-known glycoprotein, often overexpressed in several cancers and reported as a major carrier of truncated O-glycans in gastric cancer cells. The presence of these truncated O-glycans impact CD44 binding to HA, while also promoting hyperactivation of RON receptor(75). Another prominent glycoprotein in the context of cancer is MUC1, a highly glycosylated transmembrane mucin, that has also been reported to be a carrier of STn in multiple cancer types, and whose overexpression has been linked to tumor growth and metastization.

2.3 Sialylated Glycans

Aberrant glycosylation in cancer leads to the biosynthesis of tumor-associated glycans that underpin several oncogenic features leading to tumor onset and progression. Altered glycosylation encompasses the synthesis of prematurely truncated O-glycans, such as the single monosaccharide N-acetylgalactosamine (GalNAc; Tn antigen), sialyl Tn (STn) and the Thomsen–Friedenreich (T) antigen, as well as an increase in N-glycan β 1,6-branching, terminal sialylation, including the biosynthesis of polysialic acid-containing structures, and both core and antennae-linked fucosylation. These alterations support the enrichment of cell surface glycoconjugates with sialofucosylated Lewis antigens (SLe^x and SLe^a)(54) (**Figure 5**). These tumor-associated glycan structures can be present on the surface of cancer cells, but they can be also secreted or shed into the circulation and, thus, serve as potential biomarkers of disease, as mentioned anteriorly(54).

One of the main terminal modifications of glycans is the addition of a negatively charged acid (Neu5Ac) to glycoproteins and GSLs, contributing to their biophysical and biological functions. Sialylation of glycoproteins plays key roles in the blood and lymphatic vasculature, being essential for cell–cell and glycoprotein–protein interactions(76-78). The Sialyl Lewis x (SLe^x) and SLe^a isomers mediate, under physiological conditions, the binding of circulating leukocytes to cell surface selectins expressed on activated endothelial cells during immune responses(79). Frequent alterations that occur in several cancers are the increased expression of SLe^x and SLe^a. As such, tumor cells bind to vascular endothelial selectins, enhancing their migration and extravasation(80, 81). ST3Gal3, ST3Gal4 and ST3Gal6 are upregulated in different cancer types and drive

the expression of SLe^x. In GC, high expression of ST3Gal4 is associated with an increased capacity of tumor cells to invade and metastasize. In addition, SLe^x expression in tumor cells has been reported to promote proliferation, motility, angiogenesis, while protecting tumor cells from apoptosis(70).

The glycoprotein CEA is an oncofetal antigen expressed in the developing fetus and in epithelial malignancies, but only in neglectable amounts in normal adult human tissues. In gastric carcinoma, CEA was described as the main carrier of SLe^x, and this glycoform was associated with a more aggressive phenotype(82).

Apart from Neu5Ac, others non-human members of sialic acid family, named N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), although deficient or less abundant than Neu5Ac in humans, have also been detected with elevated levels in human cancers(83-85).

2.3.1 Sialyl-Tn

The STn (Neu5Ac α 2-6GalNAc α -O-Ser/Thr) antigen, also known as CD175s, is the simplest sialylated mucin-type truncated O-glycan(86). While STn expression is rarely detectable in normal tissues, high levels of this short O-glycan antigen have been widely reported across different epithelial-derived tumors (is expressed by more than 80% of human carcinomas), such as gastric, pancreatic, colorectal, and breast(74, 87, 88). STn expression has been shown to be an independent indicator of poor prognosis in carcinomas, and the detection in serum demonstrated to be useful as a biomarker for prognosis(86, 87). Later then, some works started to focus on using STn as a potential therapeutic target(88).

The mechanisms involved in STn biosynthesis include loss of C1GalT1 expression, mutations and hypermethylation of COSMC (core 1 β 3-Gal-T-specific molecular chaperone) gene, and overexpression of ST6GalNAc1 (**Figure 6**)(74, 88, 89). Expression of polypeptide-GalNAc-transferase and sialyltransferase activities are both responsible for the levels of STn expression. The efficient GalNAc-transferase activity is crucial for STn expression, since GalNAc is essential to develop the STn structure (87). Besides, overexpression of ST6GalNAc1 was correlated with STn expression in gastric and breast tumors, supporting the importance of ST6GalNAc1 in STn biosynthesis(73, 74)

STn overexpression in gastrointestinal cancer cells leads to a more aggressive phenotype, supporting the epithelial-to-mesenchymal transition (EMT) process and reducing cell–cell adhesion capacity through the loss of E-cadherin expression(90-92). Furthermore, STn-overexpressing cells are more capable of degrading and invading the

surrounding extracellular matrix (ECM), being associated with poor clinical outcome and chemotherapy resistance(90-93). It is believed that probably STn can be carried by a group of glycoproteins and have a role in protein function and in tumor development (88).

STn expression is generally reported to be heterogeneous in tumors. Various clinical features were correlated with the degree of heterogeneity of STn expression. In other words, STn expression could be regulated in the tumor, via the regulation of its carrier(s). All together, these observations suggest that STn expression could be correlated with the invasive and aggressive potential of epithelial cancer cells, when expressed at the right time and right place.

In addition, in the past the expression of STn in tumors with aberrant glycosylation of the MUC1 mucin, thus both STn and MUC1 were used as target for cancer immunotherapy in preclinical and clinical studies, especially for breast cancer(94-98). Nevertheless, STn was somewhat sidelined under the failure of the Phase III clinical trial of the Theratope (94, 95).

3. Nanoparticles for Cancer Therapy

Cancer remains one of the deadliest diseases and a major burden worldwide. Over the last decades, early detection and effective treatment of cancer have been the main focus in the field. Indeed, major advances in the fields of molecular biology and the cellular basis of cancer development and progression have been made and contributed to a more comprehensive understanding of the disease(99).

Chemotherapy comprises powerful drugs against cancer that target key biological mechanisms leading to the killing of highly proliferative cells in body. However, the use and application of these drugs in cancer treatment have significant limitations, mostly related to the dose-limiting toxicity, poor specificity towards tumor cells and the frequent emergence of drug resistance(100, 101).

Targeting cancer cells is crucial to improve the efficiency of specific drugs, avoiding drug-induced toxicity in non-neoplastic tissues, but also to aid in the detection of cancer cells and, consequently, improving diagnostics. For this reason, the development of novel and more efficient strategies to specifically target malignant cells remains of major interest (102, 103).

The current concept at the basis of precision oncology is that tumor-specific molecular abnormalities can be targeted with accurate, effective, and potentially less-toxic therapies. The first gateways for genomic accuracy in oncology were opened upon large preclinical work and primary discoveries of somatic, single-gene genomic abnormalities

underlying oncogenic transformation that could be pharmacologically targeted. The resulting aberrantly expressed proteins can be selectively targeted by strategies using specific compounds and drugs.

In recent years, nanoparticles (NPs) have been extensively explored in biomedical research. Nanomedicines have several advantages over conventional cancer therapies. Firstly, they offer greater efficiency in early detection, diagnosis, imaging, and treatment of cancers(104). Secondly, nanoscale engineering allows the production of multifunctional and “smart” NPs with extraordinary capacities, such as the ability to cross biological barriers, to target specific cells, and to control the drug release(103). NPs have been widely investigated in the cancer field due to their high sensitivity for diagnostic-based imaging but also due to constitute an alternative tool for targeted drug delivery with reduced systemic toxicity. The progress and improvements of nanosystems make them promising tools in clinical oncology(105).

The most prominent feature of therapeutic NPs is their capacity to encapsulate drugs with poor solubility, for efficiently deliver to the target tissue, with minimized off-target toxicity(105) (**Figure 7**). Indeed, specific characteristics of NPs determine nanocarrier drug delivery efficiency and biodistribution in the body (103). The most efficient way for a NP to systematically deliver a drug to the target tumor site is by remaining in circulation for a prolonged period. To do so, the nanodrug delivery efficiency can be controlled by modulating NP size and surface physicochemical characteristics (103).

Despite its known toxicity to non-cancer tissues, chemotherapy remains the most widely used therapeutic strategy in the cancer clinical setting. Doxorubicin, 5-fluorouracil and paclitaxel are known as potent anti-cancer drugs for the treatment of multiple cancers. Although these drugs efficiently impair cancer cell proliferation and growth, their systemic side effects and toxicity are also observed in healthy tissues, especially those characterized by significant cellular proliferation and turnover. For these reasons, several potent anti-tumor agents have not progressed into further clinical development (105). In this context, NPs may represent a valuable alternative to reduce a drug’s off-target adverse effects, by restricting the release of the chemotherapeutic agents to the target tumor site. Nevertheless, there are several challenges related to the use of nanotherapeutic products, such as unintended NP accumulation, as well as pharmacokinetic, physicochemical and safety profiles (106-108).

3.1 Polymeric Nanoparticles as a Strategy for Drug Delivery

Polymeric NPs are present promising strategies in nanomedicine for drug encapsulation and have gained a considerable attention due to the ability to modify their

surface (109, 110). These NPs consists of a biodegradable polymer that is known by its biocompatibility and nontoxicity. Properties such as biocompatibility are a requisite for potential application in tissue engineering, drug and gene delivery, and new vaccination strategies (111). Diverse types of polymers like alginic acid, gelatin, chitosan, poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone can be used for formulate these nanocarriers (112).

PLGA is one of the most effective and attractive polymeric NPs (113). It is approved by the European Medicine Agency (EMA) and the US Food and Drug Administration (FDA) as an ideal material for designing drug delivery systems due to controlled and sustained- release properties, low toxicity, biodegradability and biocompatibility with tissue and cells (113-115). Moreover, this polymer is widely adapted for producing NPs loading hydrophilic and hydrophobic anti-therapeutic agents (116). The drug delivery specific vehicle, in this case PLGA, should be able to deliver its payload with appropriate duration, biodistribution and concentration for the defined therapeutic effect (113). According to the promising features of PLGA, in this work we will focus in that polymer since we developed PLGA based NPs.

Nowadays, drugs and proteins are the focus for which controlled or targeted release is used to increase specificity, lower toxicity and decrease the side effects associated with treatment. Nevertheless, the stability and delivery challenges associated with these agents have complicated the number of approved products. Additionally, maintaining proper shelf-life of peptide and protein drugs often requires solid-state formulation to limit hydrolytic degradation reactions (113, 117). Thus, is crucial that the method of synthesizing NPs is successful and well done. NPs can also be synthesized by employing different methods according to the desired size and drug encapsulation. Nanoprecipitation is the most commonly method used to encapsulate hydrophobic drugs. Therefore, using the aforementioned method, polymer and drug are dissolved in acetone and then added to an aqueous solution containing Pluronic. The solvent evaporation is done under magnetic stirring at appropriate temperatures and reduced pressures leaving behind the polymer encapsulated NPs with drug(113, 118).

The safeness of PLGA NPs loaded with cytotoxic therapeutic drugs remains a huge concern and problem. The anti-cancer drugs lack capacity to targeting cancer cells and consequently will induce potent cytotoxic effects against both normal and cancer cells. Therefore, it is crucial design a PLGA nanodelivery system that, in addition to being biocompatible, biodegradable and cost-effective, can specifically release drugs at the target site with less systemic effects (116).

That's the reason that the surface modification of PLGA-NPs plays an important role in targeting strategy. When combined with polyethylene glycol (PEG), they form a block copolymer, and they increased the hydrophilicity of the formulation. From this combination, results a robust particle with enhanced blood circulation time, avoiding its immunogenicity and improving the pharmacokinetics properties of drugs by escaping opsonization (119, 120). In block copolymers, PEG chains help to orient themselves towards the external aqueous phase in micelles, thus surrounding the encapsulated species. Is also this layer of PEG that acts as a barrier and reduces the interactions with foreign molecules giving enhanced stability to the NP (113, 121). However, the addition of PEG to the system also results in decreasing the loading efficiency for drugs and proteins, even with the most appropriate methods. This reduction of drug encapsulation may be due to drug/protein-polymer interaction by the PEG chains, however, the precise mechanism for this effect remains unclear. Nevertheless, better release kinetics from formulations of block copolymers have been demonstrated in comparison to PLGA alone. Moreover, diverse mechanisms of targeted delivery of drugs from PLGA-PEG NPs have also been investigated (113, 122-127).

3.2 Decorating Nanoparticles with Monoclonal Antibodies for Selective and Efficient Cancer Cell Targeting

To design nanosystems, the intended outcome needs to consider the targeted tissue/cells. Nanocarriers can deliver drugs to tumors by two different mechanisms: passive and active targeting.

Passive targeting is accomplished by delivering NPs to defined organs via two different mechanisms, such as the reticuloendothelial system, or the enhanced permeability and retention (EPR) effect (106, 128). However, these strategies have some limitations inherent to NP physicochemical properties and the pathophysiological features of cancer tissues. Firstly, the size and surface properties of NPs need to be highly controlled to avoid both renal clearance and uptake by the mononuclear phagocyte system, both of which reduce their circulation half-life. Besides, strict control over the NP size is also important to avoid NP accumulation by the EPR effect at the tumor site, through both convection and diffusion processes. This is expected to affect NPs in the 100-400 nm diameter range (106, 108, 128). Furthermore, several chemotherapeutic drugs may induce multiple-drug resistance, which makes anti-cancer therapy become inefficient due to the resistance of cancer cells. This is a consequence of the ineffective drug delivery mechanism that lacks controlled diffusion into the tumor, accumulating NPs improperly in tissues and preventing their prolonged circulation in the bloodstream (103, 106). The passive strategy is further limited in certain hypovascularized tumors, which

do not exhibit the retention effect and are supplied by vessels depicting heterogeneous permeability and distribution. Besides, the drug delivery process by passive targeting lacks control concerning the random nature of targeting and the inefficient drug diffusion into a tumor. Thus, NPs based on active targeting can help overcome these limitations (106).

Active targeting is based on the conjugation of NPs with molecules that specifically bind to a receptor whose expression is restricted to the target cell population, while depicting neglectable expression in normal cells (103). The interaction between a ligand/mAb and its cognate receptor may induce NP internalization via receptor-mediated endocytosis and consequent intracellular drug release. Hence, non-specific interactions among NPs and normal cells can be minimized, resulting in decreased systemic side effects and increased cytotoxicity to tumor cells when applied to anti-cancer drug delivery (103, 108, 128). Obviously, the success of ligand–receptor interaction depends on the correct amount of ligand/mAb at the NP surface, ligand orientation, and target receptor expression in the target cells. In this context, increasing the number of binding sites or generating novel binding sites are considered promising strategies to overcome the current limitations of active targeting (103, 108, 128). Multiple ligands have been applied to functionalize NPs in the active targeting setting. Still, mAbs, mainly from IgG subtypes, remain the most widely used molecules to achieve active tumor targeting(106, 129).

In the early stages of development, full antibodies were used as targeting ligands. Nevertheless, multiple challenges, including immunogenicity, poor stability, fast degradation and lower efficacy further encouraged the use of antibody fragments, which allow for higher loading capacities due to reduction in crowding, superior orientation of targeting ligands and reduced variability in NP diameter constant(106, 129). NPs functionalized with an engineered human Fab targeting the alternatively-spliced CD44v6 were developed, owing to its frequent overexpression in cancer tissues. Their nanodelivery system is characterized by specificity, optimal ligand orientation and low toxicity. Finally, they produced a NP capable of strongly binding to a CD44v6-derived peptide and, more crucially, to cells with endogenous CD44v6 expression, as opposed to CD44v6-negative cells(130, 131).

The functionalization of the NPs is a critical step in the success of the targeting ability of the NP. The conjugation of the antibody to the NP could be based in carbodiimide chemistry method. This methodology consists of carboxyl groups presents in the NPs, which must be activated by the addition of cross-linking agents, such 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS). These two

reagents are added to improve the coupling efficiency to the group amine present on the antibody to be conjugated (106, 132). Group amines are the most used as functional moieties to immobilize antibodies via covalent linkage. They are abundant on the surface of the antibodies and also very reactive with a variety of functional groups on the NPs, such as carboxyl groups, without performing any chemical modification (133).

The water-soluble carbodiimide, EDC is a popular reagent used for cross-linking carboxyl or phosphate groups to primary amines (132). Besides NHS is not necessary for the carbodiimide cross-linking conducted as single-step reaction, it is usually added to the method formulation to increase the EDC-mediated coupling effectiveness. The addition of NHS provides a two-step reaction that prevents intra and intermolecular cross-linking of the antibody, since this biomolecule contains both amine and carboxyl groups (106, 134). Other point that should be considered is the activation buffer. It should not contain any primary amine or carboxyl groups, since they can disturb the EDC reaction (135). In accordance with literature, the most favorable buffer is the 2-(N-morpholino) ethanesulfonic acid (MES) buffer, while acetate and phosphate buffers can decrease the reactivity of the carbodiimide (132).

One relevant problem associated with carbodiimide chemistry is related to the presence of both carboxyl and amine groups throughout the antibody surface. To avoid this problem, excess EDC should be eliminated before adding the antibody, to restrict and minimize the possible activation of its carboxyl groups avoiding self-polymerization (106, 136).

This conjugation method is simple since the chemical modification of the antibody is not necessary. On the other hand, that missing step leads to the major disadvantage of this conjugation method. The lacking of control over antibody orientation onto the nanoparticle surface, since coupling between the functional groups and crosslinkers is not selective or controlled (134, 137).

3.3 Nanoparticles Strategies targeting Glycoproteins

Recently, the identification of aberrant glycans overexpressed by cancer cells have emerged as potential molecular candidates for the improvement of cancer targeted therapies (138, 139). As previously mentioned, aberrant protein glycosylation constitutes an incontrovertible hallmark of the malignant transformation of human cells and tissues (54, 138). Moreover, the restricted expression of glycan-based cell surface neoantigens to cancer cells makes these molecular entities promising targets for selective anti-cancer drug delivery. Over the last decade, several efforts have been made to integrate nanotechnology and carbohydrate field for the targeted and controlled

delivery of cancer therapeutic agents. Several studies have demonstrated the importance of using glycosylated NPs for therapeutic purposes (reviewed in (108, 140)).

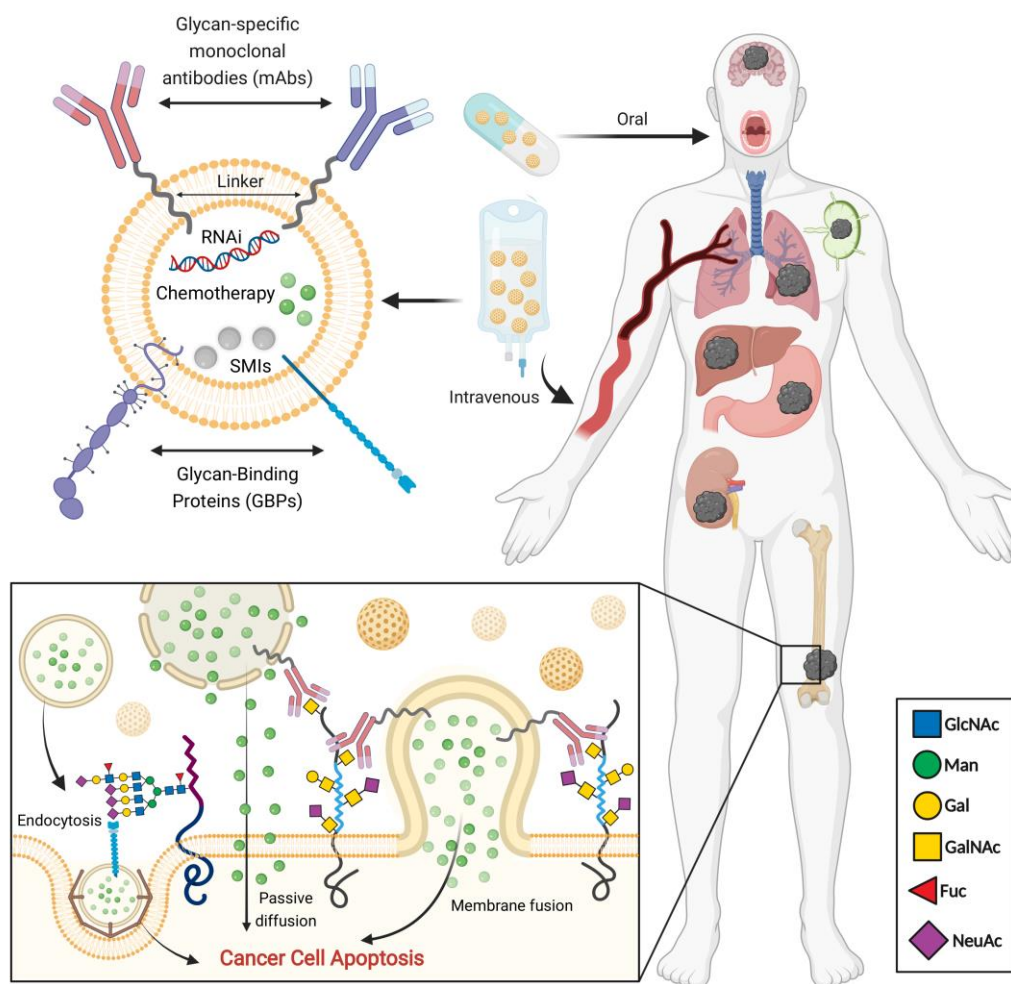


Figure 7: Strategies based on targeting cancer-associated glycans for selective and specific drug delivery systems. (Extracted from Diniz et al. 2022(59))

Vaccines Encapsulated in Synthetic Glycan-Targeting Nanoparticles with Glycan Targeting

Over the last decades, the development of vaccines has led to a significant expansion of the human life expectancy, with a positive impact on long-term protective immunity of the population (141). The mechanism underlying therapeutic cancer vaccines relies on the modulation of the host's immune response against malignancies. Cancer cells are known to express a series of tumor-associated antigens that suppress the cytotoxic activity of T cell responses. Such is the case of STn- and Tn-decorated MUC1, which is overexpressed in several malignant tumors (108).

NPs can exert optimal actions both as delivery systems and as adjuvants, with the ability to extensively enhance immune responses while ensuring minimal toxicity (142). A study conducted by Liu et al. (143) produced an anti-tumor vaccine in which a MUC1 glycopeptide was used as the target tumor-associated immunogen, the α -galactosylceramide (α -GalCer) acted as an immune adjuvant, and gold-based NPs (AuNPs) as the multivalent carriers. The developed vaccine triggered a significant and durable antibody response, through specific binding of immunized serum to MUC1-expressing breast cancer cells. In another study, Cai et al. (144) developed a vaccine in which MUC1 glycopeptides were covalently linked to CD4 T-cell peptide epitopes and polyethylene glycol (PEG) combined with AuNPs. This formulation was able to promote antigen presentation by antigen presenting cells, modulating T-cell activation and MHC II-restricted T- and B-cell cooperation. Finally, Mocan et al. (145) demonstrated that AuNPs functionalized with a MUC1 protein fragment generated efficient anti-cancer responses via macrophage activation and polarization towards an inflammatory phenotype (M1). A polymerizable version of the Tn antigen was conjugated to AuNPs and was able to induce the strong and durable production of anti-Tn antibodies, demonstrating that the efficiency of glycan-directed vaccines is comparable to that of formulations targeting entirely peptidic antigens (146).

More recently, a novel antigen delivery system using MUC4, a glycoprotein highly overexpressed in pancreatic tumors, was decorated with the T antigen at different sites. Their synthetic vaccine targets antigen-presenting cells (APC) expressing dectin-1, a C-type lectin known to bind β 1,3-glucans. The obtained results showed that these particles induce strong in vivo immune responses, such as production of antibodies and the activation and proliferation of antigen-recognizing T-cells (147).

Some studies also focus on the new strategy based on the selective targets of the mannose receptor expressed in APC, such as dendritic cells and macrophages. It demonstrated a simultaneous enhancement of antigen-specific CD4⁺ and CD8⁺ T-cell responses by mannan-decorated poly(lactic-co-glycolic-acid) (PLGA) NPs with ovalbumin encapsulated, in comparison with non-decorated NPs (148). In addition, drug-free mannosylated liposome NPs were also used to inhibit tumor growth by promoting the polarization of tumor-associated macrophages. It was shown that the induction of the M1 phenotype is associated to the increase of antitumor immune efficacy of immunomodulators (149).

Nanoparticle Strategies targeting Glycoproteins

Several reports demonstrated the use of aptamers to target carbohydrate moieties, such as Neu5Ac, instead of mAbs (104, 150-153). Aptamers are synthetic oligonucleotides or peptide molecules with a small size, lower production cost, high specificity, and reduced immunogenicity(154). A recent study (155) has developed an anti-MUC1 aptamer conjugated to a chitosan-based NP to co-deliver insulin-like growth factor receptor 1 (IGF-1R) siRNA and the docetaxel chemotherapeutic. This nanoformulation was conjugated with an anti-MUC1 aptamer and was shown to efficiently bind to MUC1-overexpressing metastatic breast cancer cells. Moreover, the developed NP was shown to be efficiently internalized by malignant cells and produce the desired downregulation of IGF-1R expression. Sayari et al. (151) have developed a similar nanocarrier system for the delivery of SN38, the active metabolite of irinotecan, to colon cancer cells. The poor solubility and high toxicity of SN38 have so far precluded its clinical use. However, the encapsulation and specific delivery of this compound to cancer cells may help overcome both these limitations. A MUC1-directed aptamer has been further conjugated with a mesoporous silica NP. The NP was loaded with epirubicin, a cytotoxic drug commonly used to treat breast cancer in the adjuvant setting, and depicted increased uptake and cytotoxicity in breast cancer cells (152). Perepelyuk et al. evaluated the therapeutic efficacy of MUC1-aptamer functionalized hybrid NPs in lung tumor-bearing mice. This hybrid nanosystem was loaded miRNA-29b, a microRNA targeting DNA methyltransferases, and was shown to increase its specific delivery to the target cells and tissues (153). Lastly, Yu et al. (150) also reported the potential of a MUC1 protein aptamer when as a targeting agent. Paclitaxel was loaded in aptamer-conjugated NPs of PLGA and validated in a MUC1-overexpressing breast cancer cells.

CD44 constitutes an additional appealing molecular candidate for anti-cancer targeted drug delivery(156). One study has developed NPs coated with HA-appended PEG-PLGA polymers for the specific targeting of CD44-expressing triple-negative breast cancer cells. Such formulation, loaded with antisense miR-34a, depicted enhanced stability and potent in vivo toxicity (157). A similar approach has been developed in the bladder cancer context, using CD44-targeting NPs loaded with anti-Bcl2 siRNAs (158). Furthermore, PLGA-based NPs decorated with a Fab recognizing the CD44v6 variant highly expressed by gastrointestinal cancer cells have been developed (159).

Some other studies have employed methods to evaluate CEA, a cell surface glycoprotein overexpressed in several tumors, as a therapeutic target. A strategy was created for the targeting of triple-negative breast cancer using CEA-directed iron NPs, which depicted

an enhanced tumor targeting capacity (160). Additionally, CEA-targeting PLGA-PEG NPs loaded with paclitaxel have been demonstrated to selectively bind and trigger the death of CEA-expressing colorectal cancer cells (125).

Glycan-Targeting Nanoparticles

Targeting cancer-associated glycan epitopes is another promising strategy to achieve targeted drug delivery in cancer tissues. A commonly targeted glycan is SLe^a, due to its high expression during malignant transformation. In this context, functionalized PLGA NPs with a mAb targeting SLe^a and loaded with paclitaxel and 5-FU have been developed (126). More recently, Palma-Chavez et al. developed and characterized a PLGA-based multistage delivery system for improved drug delivery to SLe^a-expressing inflamed vascular endothelium(161). Other frequently targeted glycan, due to its high expression and exclusivity during cancer disease is STn. Regarding this, Gidwani et al. (162) developed STn-monoclonal antibodies coated on europium NPs that were used for diagnostic purposes in cancer. We also demonstrated the efficient ability of targeting and specificity of NPs functionalized with anti-STn mAb and loaded with LPT in GC cells, during static and fluidic conditions (124).

Nanoparticles Strategies Using Glycan-Binding Proteins

GBPs regulate the crosstalk between distinct sets of immune cell populations and tumor cells. By actively supporting the establishment of an immunosuppressive microenvironment, GBPs have emerged as promising targets of more efficient immunotherapies. Several studies have developed NPs functionalized with a mAb targeting Siglec-3 (CD33), which is abundantly expressed on the surface of acute myeloid leukaemia cells (163-166). Furthermore, novel bi-specific liposomal-based NP T cell engagers (nanoTCEs) are conjugated with two mAbs targeting two distinct epitopes, one being a cancer antigen, and the other the Siglec-3 (163). This formulation has shown to be efficient in the targeting and killing of AML cells, both *in vitro* and *in vivo*.

Another attractive target is Siglec-1 (CD169/sialoadhesin), due to its ubiquitous expression on macrophages. Chen et al. described a strategy using liposomal NPs with high binding affinity towards glycan-based ligands of Siglec-1 (167).

Several studies have been conducted using NPs functionalized with carbohydrate-recognizing lectins(168-172). For instance, the mannose-binding concanavalin A was used to functionalize NPs and target sialic acids (168, 172). In addition, the conjugation of the wheat germ agglutinin to NPs loaded with anti-tumor chemotherapeutics has been shown to significantly enhance cancer cell-specific uptake, while minimizing off-target

cytotoxicity (169). Similar strategies have been employed for the targeting of cancer cells enriched in fucosylated antigens, such as Lewis x (Le^x), using the fucose-recognizing lectins *aleuria aurantia* lectin (170) and the *lotus tetragonolobus* lectin (171).

AIMS

1. Rationale and General Aim

Gastric cancer is one of the most common malignancies with poor prognosis and high mortality. Regarding the current efforts of personalized cancer treatment, tyrosine kinase inhibitors are among the most promising medicines. Cell glycosylation modulates receptor's activation and impairs the outcome of cancer therapy.

To optimize delivery and minimize the toxicity of drugs, a strategy based on the knowledge of a specific expression of Sialyl-Tn, a glycan aberrantly expressed in gastric tumors will be used to develop nanoparticles targeting this glycan. The efficiency of the decorated nanoparticles containing tyrosine kinase inhibitors will be validated both *in vitro* using the 3D-glyco-engineered models and in an *in vivo* mouse model.

Thus, the general aim of the present work is to develop a novel nanosystem that consists in polymeric nanoparticles loaded with different TKIs functionalized with an antibody directed to Sialyl-Tn.

2. Specific Aims

Specific Aim 1 – Chapter 2 - Development of a polymeric nanoparticles targeting Sialyl-Tn in gastric cancer:

The aim of this Chapter was the development of nanoparticles surface-functionalized with a specific antibody targeting the Sialyl-Tn glycan and further evaluate this nanosystem effectiveness regarding its specificity and recognition capacity. Following this, we intended to show the loading capacity of the polymeric nanoparticles with Lapatinib and to demonstrate the cytotoxic effect in gastric cancer cell lines. Lapatinib is a potent multitargeted tyrosine kinase inhibitor, directed to EGFR and HER2. Finally, our last goal was to validate our nanosystem under flow conditions with a live tracking system. The study of interactions between nanoparticles and cells under flow conditions is crucial to evaluate the cellular responses in physiological-like environments. In terms of NPs applicability, flow stimulation can critically affect the efficacy of the targeting or the drug delivery accumulation in the tumor region. Contrarily to under flow conditions, static culture promotes the sedimentation of nanoparticles, supporting the local accumulation of nanoparticles and resulting in unspecific binding to the target cells. This new approach that we developed to study the nanoparticles interaction with cells under live flow conditions, provided the opportunity to track the nanoparticles for a time period and analyze their interaction with the target cell.

Specific Aim 2 – Chapter 3 – Evaluation of nanoparticles targeting Sialyl-Tn as an approach for the delivery of a tyrosine kinase inhibitor in Gastric Cancer:

In this Chapter we proposed to load with Foretinib, polymeric nanoparticles, functionalized with an antibody directed to Sialyl-Tn. Foretinib is an oral multikinase inhibitor targeting MET and RON receptors tyrosine kinases, that has been used in clinical trials for several solid tumors. Then, we aimed to show the effect of these nanoparticles in receptors phosphorylation activation of multicellular tumor spheroids of gastric cancer Sialyl-Tn overexpressing cells. Multicellular tumor spheroids have been previously shown to be a good model to better mimic the tumor microenvironment and help fill the gap between 2D cell culture and *in vivo* models. Lastly, we developed Sialyl-Tn overexpressing gastric cancer xenograft mice models and validated the delivery of our nanoparticles to demonstrate a reducing effect on tumor volume, cell proliferating and tumor necrosis area due to the treatment with nanoparticles functionalized with antibody anti-Sialyl-Tn.

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CHAPTER 2

Paper I

Polymeric nanoparticles targeting Sialyl-Tn in gastric cancer: A live tracking under flow conditions

Francisca Diniz, Maria Azevedo, Flávia Sousa, Hugo Osório, Diana Campos, Paula Sampaio, Joana Gomes, Bruno Sarmento,

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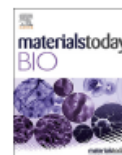
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Polymeric nanoparticles targeting Sialyl-Tn in gastric cancer: A live tracking under flow conditions

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ABSTRACT

Drug delivery using nanoparticles (NPs) represents a potential approach for therapy in cancer, such as gastric cancer (GC) due to their targeting ability and controlled release properties. The use of advanced nanosystems that deliver anti-cancer drugs specifically to tumor cells may strongly rely on the expression of cancer-associated targets. Glycans aberrantly expressed by cancer cells are attractive targets for such delivery strategy. Sialylated glycans, such as Sialyl-Tn (STn) are aberrantly expressed in several epithelial tumors, including GC, being a potential target for a delivery nanosystem. The aim of this study was the development of NPs surface-functionalized with a specific antibody targeting the STn glycan and further evaluate this nanosystem effectiveness regarding its specificity and recognition capacity. Our results showed that the NPs surface-functionalized with anti-STn antibody efficiently are recognized by cells displaying the cancer-associated STn antigen under static and live cell monitoring flow conditions. This uncovers the potential use of such NPs for drug delivery in cancer. However, flow exposure was disclosed as an important biomechanical parameter to be taken into consideration. Here we presented an innovative and successful methodology to live track the NPs targeting STn antigen under shear stress, simulating the physiological flow. We demonstrate that unspecific binding of NPs agglomerates did not occur under flow conditions, in contrast with static assays. This robust approach can be applied for *in vitro* drug studies, giving valuable insights for *in vivo* studies.

1. Introduction

Gastric cancer (GC) represents the fifth most incident and the third most lethal cancer worldwide with an incidence of 1 million new cases per year [1]. This high mortality of GC is associated with a late diagnosis, as the patient becomes symptomatic in an advanced stage, limiting the therapeutic options and the efficacy of treatment [2]. Recent approaches applying nanotherapeutics are recognized as potential strategies to improve patient treatment [3]. The use of nanoparticles (NPs) that are capable of remaining in the blood circulation for an extended time period and that can be engineered to target cancer cells represent a major opportunity to increase therapy efficacy. NPs need to avoid nonspecific

interactions with healthy cells and circumvent *in vivo* barriers to avoid side effects and loss of drug-carrying NPs. The control of the NPs surface constituents is crucial for the determination of their *in vivo* interaction and optimization of the nano-drug delivery efficiency [4–6]. Moreover, targeting cancer cells with functionalized NPs carrying anti-cancer drugs allows an efficient therapy reducing the major side effects commonly caused by drugs used in oncology [3,4,7].

Poly (lactic-co-glycolic acid) (PLGA) is a widely used polymer for production of NPs due to its biocompatibility and biodegradability, long-standing track record in biomedical applications and well-documented utility for sustained drug release [8,9]. For that it was approved by US Food and Drug Administration (FDA) and European Medicines Agency

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(EMA) for medical applications [10]. The surface modification of PLGA-NPs plays an important role with respect to targeting strategy. When combined with polyethylene glycol (PEG) they increased the hydrophilicity of the formulation resulting in a robust particle with enhanced blood circulation time, avoiding its immunogenicity and improving the pharmacokinetics properties of drugs by escaping opsonization [8,11].

Despite the advances of nanotherapeutics in medicine, there is still major limitations regarding cancer cell targeting and drug delivery. To improve the specificity of the NPs, defined antibodies targeting ligands such as carbohydrates antigens aberrantly expressed by cancer cells might be attached to the surface of NPs by functionalization [12]. The aberrant biosynthesis of glycans are common features in cancer leading to the display of specific cancer-associated glycan structures with important roles in tumor progression and metastization [13–15]. Moreover, the use of glycans as targets for cell delivery has been shown to be an effective strategy for cell-selective drug delivery [7]. NPs functionalized with antibodies or molecules targeting carbohydrates would provide a largely multi-valent technique of interactions with target cells showing increased local ligands level on design surfaces [4,7,12].

Sialyl-Tn (STn), a truncated O-glycan containing sialic acid residue, is aberrantly expressed in several epithelial cancers, including GC [15–18]. This antigen is not expressed in normal tissues, whereas it is highly expressed in carcinomas and associated with aggressive tumors such as those with chemotherapy resistance and poor prognosis [18–20]. The biosynthesis of the STn antigen is dependent on the expression of the sialyltransferase ST6GalNAc1 [18,21], as well as to the mutations of the gene encoding the molecular chaperone COSMC [22,23]. We previously showed that STn plays a critical role during cancer progression impairing clinical outcome and leading to poor prognosis and a more aggressive phenotype in GC patients [18,24]. STn-monoclonal antibodies coated on europium NPs were previously used for diagnostic purposes in cancer [25]. Given its expression and association with GC malignant features, the STn antigen has a higher potential as a target for a new NP system using an antibody directed to STn.

The study of NP-target cell interactions under flow conditions is crucial to evaluate the cellular responses in physiological-like environments. In terms of NPs applicability, flow stimulation can critically affect the efficacy of the targeting or the drug delivery accumulation in the tumor region [26,27]. Thus, the physiological flow stimulation is important to mimic the *in vivo* conditions for NPs risk management. Contrarily to under flow conditions, static culture promotes the sedimentation of NPs, supporting the local increase in the NPs concentration, and resulting in unspecific binding to the target cells [28–30]. In addition, it has been reported that flow can affect cell-NP interactions and therefore the NP uptake via the cells. Accordingly, rates of NPs exocytosis and endocytosis by cells have been observed with slight differences upon perfusion [27,31,32]. Thus, the cell's response under flow should be considered for improved validation of the targeting.

Here, we developed a nanodelivery system functionalized with an antibody against STn and we further evaluate their interaction with cells and ability to target the tumor glycan in both static and under flow conditions. For this purpose, we applied a perfusion system in a controlled microenvironment to better understand the interaction of the developed targeted polymeric NPs with glycoengineered cells expressing STn antigen, under a flow-induced shear stress. This new tool to study the effect of the NP interaction with cells under live flow conditions, provide the opportunity to track the NPs for a time period and analyze their interaction with the target cell.

2. Material and methods

2.1. Synthesis of PLGA-PEG-COOH nanoparticles

The Lapatinib-loaded PLGA-PBG-COOH NPs were prepared by using nanoprecipitation method [33,34]. PLGA-PEG-COOH block copolymer

was used to present the carboxylic acid on the NP surface, allowing the carbodiimide reaction to amine group from the antibody. Briefly, 19 mg of Poly(lactide-co-glycolide) (PLGA) 5004A (provided by Corbion) and 1 mg of Poly(lactide-co-glycolide)-*b*-poly(ethylene glycol)-carboxylic acid endcap (PLGA-PEG-COOH) (PolySciTech) were added to 1 mg of Lapatinib (Sigma-Aldrich, Ref. CDS022971) (dissolved in DMSO, 20 μ l) and left in 1,980 mL of acetone (ThermoFisher) to allow the complete dissolution. Further, this organic solution was transferred using a 25G needle to an aqueous solution of 0.5% (w/v) Pluronic F-127 (Sigma-Aldrich) (20 mL) under magnetic stirring (300 rpm) for 3h and the remaining organic solvent was removed by evaporation. Finally, NPs were washed with MilliQ water, using 100 kDa Amicon® filters, centrifuged for 20min at 2,000g, thrice. The NPs were resuspended in 1.5 mL of PBS in the final. The same nanoprecipitation method was also used to produce Lapatinib-loaded PLGA and empty NPs without the presence of PLGA-PEG-COOH and Lapatinib. Fluorescent NPs were produced to allow NP tracking under flow assays, following the same nanoprecipitation method described in a previous study [32]. Briefly, it was added 2 mg of Poly(lactide-co-glycolide)-Fluorescein (PolySciTech), that corresponds to 5% of PLGA-FITC of the theoretical mass. As the FITC conjugation is covalent, early release of the fluorophore from NPs is not expected.

2.2. Nanoparticle-antibody conjugation

Monoclonal antibody (mAb) B72.3 (anti-STn) [35] was purified from hybridoma culture media using the HiTrap™ Protein G HP 1 mL columns of purification (Sigma-Aldrich). The conjugation of NPs with anti-STn (B72.3) or IgG1 Isotype Control Purified Mouse Monoclonal IgG antibody (R&D Systems) was performed via carbodiimide chemistry which allowed to chemically link the antibody ligand to the COOH group of PLGA-PEG present on the surface of NPs. NPs (150 μ l, 1:10 of the final NP concentration 14 mg/mL) were resuspended in 820 μ l of 2-(N-morpholino) ethanesulfonic acid (MES) buffer (0.1 M, pH 5.5). Then, were incubated with 27 μ l of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (22 mg/mL) and 3 μ l of N-hydroxysuccinimide (NHS) (8 mg/mL) for 30 min at room temperature (RT), protected from light and with gentle stirring. NPs were washed twice with PBS (centrifuged at 5000g for 8min, RT), the last resuspension was performed in 500 μ l of PBS. The resulting activated NPs were reacted with 33 μ g of B72.3 or IgG1 antibody, resuspended in 500 μ l of PBS, for 2h at room temperature and gentle stirring. Further, the functionalized NPs were washed twice using PBS and kept at 4 °C in 1 mL PBS until use. These last two washes were kept to be quantified further in conjugation efficiency evaluation by indirect method.

2.3. Physicochemical characterization of the formulations

After washing, all the formulations were physicochemically characterized regarding the particle size (nm), polydispersity index and surface charge (ζ -potential in mV) using Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd). The NPs before measuring were diluted in sodium chloride (NaCl) 10 mM, pH 7.4.

2.4. Morphology of nanoparticles

The morphology of the NPs was observed through Transmission Electron Microscopy (TEM). Briefly, 10 μ l of each NPs sample were mounted on nickel grids and left to stain for 2 min approximately. Afterwards, the excess liquid was removed with filter paper, and a Uranyl Acetate solution (1%) was added and left to incubate for 10s. Samples were viewed on a JEOL JEM 1400 TEM (JEOL, Tokyo, Japan) operated at 80 kV, and images were digitally recorded using a CCD digital camera Orius 1100W (Tokyo, Japan).

2.5. Association efficacy, drug loading and conjugation efficiency

2.5.1. LC-MS

The association efficacy and drug loading of Lapatinib loaded in NPs was determined by LC-MS. NPs formulations were exposed overnight at freeze dryer and then were resuspend in dimethylformamide for drug extraction. The LC-MS analysis was done in an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% AcCN, 0.1% FA at 30 µL/min. After 1 min loading, the trap column was switched in-line to a EASY-Spray nano Flow Emitter with 10 µm inner diameter without transfer line (ES993, Thermo Scientific, Bremen, Germany) at 1 µL/min. Separation was achieved by mixing A: 0.1% FA and B: 80% AcCN, 0.1% FA with the following gradient: 1 min (2.5% B to 99% B), 3 min (hold 99% B). Subsequently, the system was equilibrated with 2.5% B. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380–1300) and subsequent HCD MS/MS of the 5 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chrom. peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), AGC target 3×10^6 , maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3 , intensity threshold 7.3×10^4 , charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 5 s. The MS2 settings were as follows: microscans 1, resolution 35 k (m/z 200), AGC target 2×10^5 , maximum injection time 110 ms, isolation window 4.0 m/z , isolation offset 0.0 m/z , dynamic first mass, and spectrum data type profile.

Association Efficacy (%) = (Mass of the drug in NPs / Initial Mass of drug in the NPs) × 100

Drug loading capacity (%) = (Mass of the drug in NPs / Initial Mass of the NPs) × 100

We also performed LC-MS to confirm the conjugation efficiency of B72.3 or IgG antibody to NPs, conjugated with Lapatinib, by direct method. Proteomic sample preparation and data acquisition was performed as previously described [36]. Data analysis was carried out with the software Proteome Discoverer version 2.5.0.400 (Thermo Scientific). Protein identification analysis was conducted by the Sequest node using the SwissProt Protein database (2020_05, 565,553 entries). Protein Label Free Quantitation was performed by the Minora and Precursor Ions Quantifier nodes.

Conjugation Efficiency (%) = (Mass of antibody in NPs / Initial Mass of antibody in the NPs) × 100

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

The binding efficiency of B72.3 antibody and IgG1 control to NPs was also quantified by indirect method using enzyme-linked immunosorbent assay (ELISA). ELISA was performed using 96-well MaxiSorp plates (Nunc, Denmark). Plates were coated overnight at 4 °C with 10 µL of supernatant of each sample of NPs or the positive control sample (same amount of antibody used in the functionalization process) in bicarbonate-carbonate buffer (pH 9.6) and blocked in PLI-P (PO4, Na/K, 1% Triton X-100 (Sigma-Aldrich), 1% BSA) buffer (pH 7.4) for 1 h at RT. Following washing, plates were incubated with Goat anti-mouse HRP antibody (1:5000) (Jackson ImmunoResearch) for 1 h at RT. Plates were developed with TMB + substrate (Thermo Scientific), reactions were stopped with

0.5 M H₂SO₄ (VWR), and absorbance was read at 450 nm using SynergyMx™ Multimode Microplate Reader (BioTek™, USA). All samples were done in triplicate. The obtained absorbance values were used to calculate the conjugation efficiency (%).

Conjugation Efficiency (%) = ((Absorbance of the input – Absorbance of the sample) / Absorbance of the input) × 100

2.6. ImmunoTEM

The antibody bound to NP was visually confirmed by ImmunoTEM. Briefly, approximately 10 µL of NPs were mounted on nickel grids and left to stain for 20 min. Then 1% BSA, PBS was added for 30 min at room temperature to block any unspecific signal. Afterwards, gold goat anti-mouse (1:50) (Abcam) was added for 20 min. Followed by 6 washes with PBS and 6 washes with distilled water, 3 min each. Finally, 5 µL of Uranyl Acetate solution (1%) was added and left to incubate for 10 s. Samples were viewed on a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV, and images were digitally recorded using a CCD digital camera Orius 1100W (Tokyo, Japan).

2.7. Cell lines and cell culture reagents

Gastric carcinoma cell line MKN45 (MKN45 WT) and AGS (AGS WT) were obtained from the Japanese Cancer Research Bank (Tsukuba, Japan) and the American Type Culture Collection (Manassas, VA, USA), respectively. The MKN45 SimpleCell (MKN45 SC) and AGS SimpleCell (AGS SC) models were generated by targeting the C1GALT1 (COSMC) gene using zinc-finger nuclease precise gene editing, which results in knockout COSMC, as previously described [37]. Cells were grown in monolayer culture in cell culture flasks, maintained at 37 °C in an atmosphere of 5% CO₂, in RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Biowest, Riverside, MO, USA).

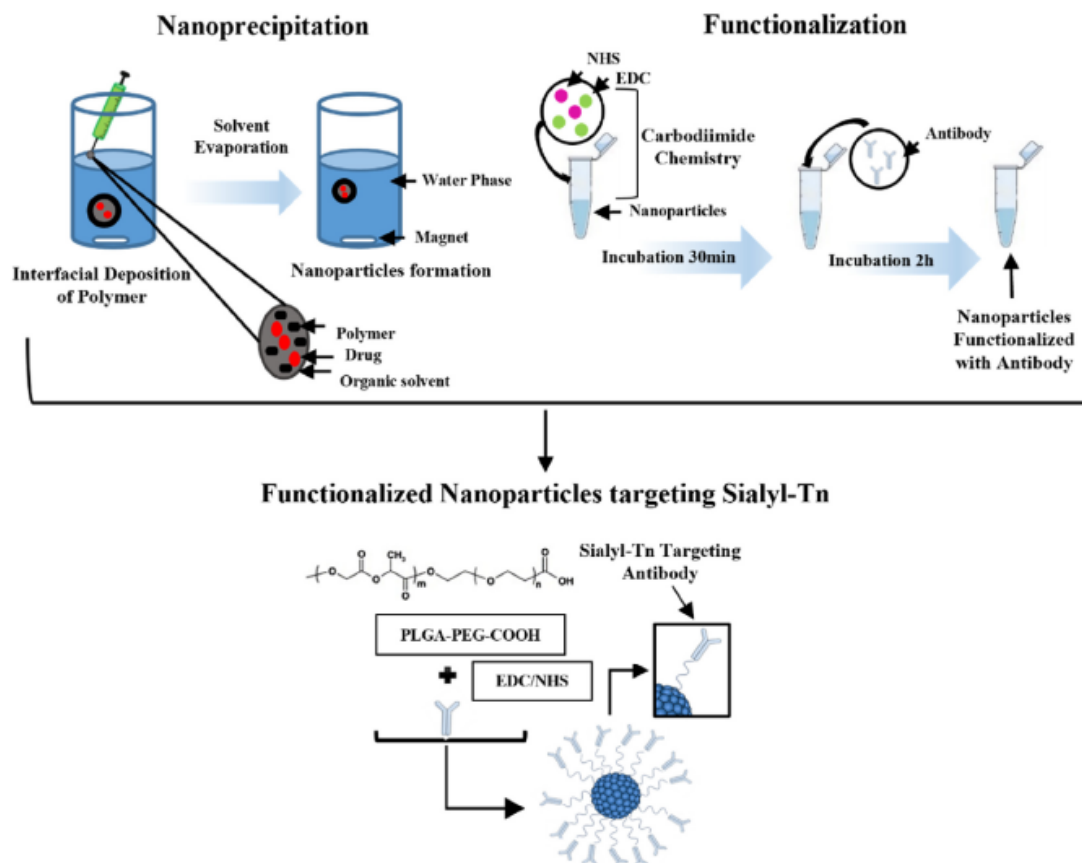
2.8. Cell-nanoparticle interaction studies under static conditions

2.8.1. Flow cytometry

MKN45 WT and MKN45 SC cells were seeded in 6-well plates 48 h before analysis. Cells were detached using Versene solution (Thermo-Fisher), and 1 million cells were used in each condition. Cells suspension was fixed with 2% Paraformaldehyde (PFA) (Alfa Aesar), PBS for 20 min at RT, and then washed twice with PBS at 350g for 5 min. Cells were permeabilized with 0.1% Triton, PBS for 5 min at 4 °C and washed twice with PBS. Afterwards, cells were incubated under gentle agitation at RT with functionalized NPs (100 µg/mL), B72.3 antibody (33 µg) or IgG1 antibody (33 µg) for 2 h. After, cells were washed and incubated with the secondary antibody goat anti-mouse Alexa Fluor® 488 (1:200) (Invitrogen) for 30 min in the dark. Cells were washed and resuspended in PBS, strained and labelled with DAPI (2 µg/mL) (Sigma-Aldrich). The same method was used for samples previously treated with the neuraminidase enzyme (0.2 U/mL) (Sigma-Aldrich) diluted in simple medium during 1 h 30 min at 37 °C. Cells were incubated with B72.3 functionalized PLGA-PEG NPs (100 µg/mL) and monoclonal antibody to the Tn antigen (5F4) (undiluted) [38]. Cells suspension was analyzed using BD FACS Canto™ II (BD Biosciences, San Jose, CA, USA). Three independent experiments were conducted. Data were analyzed using FlowJo (BD Biosciences, San Jose, CA, USA).

2.8.2. Immunofluorescence

A defined concentration of 4×10^4 cells/mL of MKN45 WT and MKN45 SC were seeded in a 12-well standard microscope glass slide with a removable silicone chamber (Ibidi GmbH, Munich, Germany) and left



Scheme 1. Schematic representation of methodology behind the development of encapsulated and functionalized nanoparticles.

in the incubator for 48h. Cells were exposed during different time points (0h, 2h, 4h and 6h) to both functionalized NPs independently. After that were washed twice with PBS and fixed with 4% PFA for 15min followed by permeabilization with 0.1% Triton X-100-PBS for 5min at 4 °C. Blocking was done with Goat serum (1:5) (Dako) in 10% BSA-PBS at room temperature for 30min. Goat anti-mouse Alexa Fluor® 488 secondary antibody (1:200) (Invitrogen) and Alexa Fluor 568 Phalloidin (1:40) (ThermoFisher) were diluted in 5% BSA, PBS and PBS, respectively, and cells were incubated for 30min. Nuclear stain was done with DAPI (1:100) in PBS for 10min and the preparation was mounted using Vectashield. Imaging was performed on a Zeiss AxioImager Z1 (Carl Zeiss, Oberkochen, Germany) equipped with a AxioCam MR ver3.0 (Carl Zeiss, Germany), and an HXP 120 light source (metal-halide lamp) using a Plan-Apochromat 20x/0.8 or a Plan-Apochromat 40x/1.30 Oil objective. For DAPI, Alexa Fluor 488 and Alexa Fluor 568 phalloidin acquisition, the following filter cube sets were used, respectively: Excitation: G 365 Beam Splitter: FT 395 Emission: BP 445/50; Excitation: BP 470/40 Beam Splitter: FT 495 Emission: BP 525/50 and Excitation: BP 450–490 Beam Splitter: FT 510 Emission: BP 515–565.

2.8.3. Metabolic activity assay

Metabolic activity was measured using a resazurin reduction assay. Cells were seeded in triplicate in a 96-well plate at a density of 1×10^4

cells/well and treated with 100 µg/mL of functionalized and non-functionalized NPs. Cells treated with hydrogen peroxide (H_2O_2) and untreated cells were used as positive and negative controls, respectively. After 5, 5h and 24h, 0.02 mg/ml resazurin (Sigma-Aldrich) was added to the cells and incubated for additional 2h at 37 °C. Fluorescence intensity (ex. 530 nm; em. 590 nm) was measured using a Synergy™ Mx microplate reader (Agilent, Santa Clara, CA, USA).

The same experimental protocol was used to measure the metabolic activity of the cells after 24h and 48h of treatment with 1 µg/mL of free Lapatinib, IgG Functionalized Lapatinib-loaded PLGA-PEG NPs (NLIG) and B72.3 Functionalized Lapatinib-loaded PLGA-PEG NP (NLB72.3).

2.9. Cell-nanoparticle interaction studies under flow conditions

The experiments under flow were performed using a peristaltic pump system (ISMATEC, model ISM931C). Initially, 7×10^5 cells/channel were seeded in a 6-channel polymer µ-slide suitable for flow assays (µ-Slide VI 0.4, Ibidi GmbH, Munich, Germany), previously coated with N-Poly-L-Lysine (PLL) (Sigma-Aldrich) (10 µg/mL). Cells were grown for 72h, until a confluent cellular monolayer was formed. Afterwards, the medium was replaced to phenol-red-reduced RPMI 1640 medium (Gibco) supplemented with 10% FBS, 25 mM HEPES buffer (Gibco) solution, 2 mM GlutaMAX (Gibco), 1% Sodium Pyruvate (Cytiva) and 1%

Table 1

Properties of functionalized and non-functionalized nanoparticles, including the mean size, polydispersity index (PDI), surface charge and conjugation efficacy (CE). The values are represented as mean values $\pm$ SD (N = 3). Non applicable (n.a.).

Nanoparticle Sample	Size (nm)	PdI	Surface Charge (mV)	CE (%) ELISA
PLGA NP (NP)	152.8 $\pm$ 2.3	0.087 $\pm$ 0.018	-12.7 $\pm$ 0.8	n.a.
PLGA-PEG-COOH NP (NPP)	147.1 $\pm$ 0.6	0.055 $\pm$ 0.009	-15.8 $\pm$ 1.2	n.a.
B72.3 Functionalized PLGA-PEG NP (NB72.3)	179.4 $\pm$ 2.5	0.174 $\pm$ 0.024	-16.0 $\pm$ 1.3	85.3 $\pm$ 0.3
IgG Functionalized PLGA-PEG NP (NIG)	178.8 $\pm$ 2.2	0.161 $\pm$ 0.028	-14.9 $\pm$ 1.9	75.9 $\pm$ 0.4
B72.3 Adsorbed PLGA-PEG NP (NAdB72.3)	186.7 $\pm$ 1.5	0.139 $\pm$ 0.024	-18.3 $\pm$ 0.1	64.1 $\pm$ 7.5
FTIC-labelled NP (NF)	140.7 $\pm$ 1.5	0.117 $\pm$ 0.019	-12.4 $\pm$ 0.2	n.a.
B72.3 Functionalized PLGA-FTIC-PEG NP (NF-B72.3)	214.5 $\pm$ 6.4	0.189 $\pm$ 0.037	-12.4 $\pm$ 1.5	88.5 $\pm$ 3.3
IgG Functionalized PLGA-FTIC-PEG NP (NF-IgG)	217.6 $\pm$ 8.2	0.191 $\pm$ 0.046	-12.6 $\pm$ 1.2	80.1 $\pm$ 5.9

Penicillin-Streptomycin (100x) (Gibco). Therefore, 100 μ g/mL of respective NPs were diluted in 6.5 mL of cell culture medium. Each channel of the μ -slide was connected to the tubes and to the reservoir containing the medium. Cell binding studies under flow conditions were performed live at a shear stress of 2 dyn/cm², for 6h. During the optimization process, we also tested 10 dyn/cm² and 5 dyn/cm², however we selected 2 dyn/cm² since this represented the best option for the viability of our cells and was in accordance with what was previously described in the literature (venous system associated to a shear stress between 1 and 6 dyn/cm²). Images were acquired every 15min, using an inverted motorized Leica DMI6000 (Leica Microsystems) widefield microscope, equipped with a Hamamatsu Orca-FLASH 4.0 sCMOS camera (C11440, Hamamatsu), an HCX PL FL L 40x/0.60 CORR Ph2 objective, a Leica EL6000 light source (metal-halide lamp), and controlled through the LAS X software (version 3.5.5.19976). Samples were imaged using binning 2 $\times$ 2. Alexa Fluor 488 beads acquisition was done using the following

filter cube set: Excitation: 460/40; BS: 505; Emission: 527/30. The video and image analysis were performed using ImageJ software [39].

2.10. Statistical analysis

Results are expressed as mean $\pm$ standard deviations (SD) of three independent experiments. Data were analyzed using Student's t-test for the individual group comparison, with GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). The level of significance was set at probabilities of *p < 0.05; **p < 0.01 and ***p < 0.001.

3. Results and discussion

3.1. Production and characterization of the polymeric nanoparticles

In this study, NPs were produced by nanoprecipitation (Scheme 1), a commonly used method to encapsulate hydrophobic drugs in polymeric NPs. Then we proceeded to the functionalization of the NPs process (Scheme 1), a critical step in the success for the conjugation of the antibody to the NP based in carbodiimide chemistry method. This conjugation consists of carboxyl groups of the NPs, which must be activated by the addition of cross-linking agents, such EDC, and NHS. These two compounds, are added to improve the coupling efficiency to the group amine present on the antibody to be conjugated (IgG1 or B72.3) [40,41]. PEG was used as it has been shown to prolong NP circulating half-time which being associated with reducing nonspecific adsorption of biomacromolecules and contributing to decrease of nonspecific interactions [8].

Physical-chemical properties of NPs were assessed such as their size, surface charge and size distribution and summarized in Table 1. The size of the different formulations was ranged between 140 nm and 217 nm, with a polydispersity index (PdI) lower than 0.2, indicating a relative homogenous size distribution. All formulations presented negative surface charge, between -18 and -12 mV.

The analysis of the developed NPs by TEM showed that the surface morphology where spherical and they were well distributed (Fig. 1, A-D).

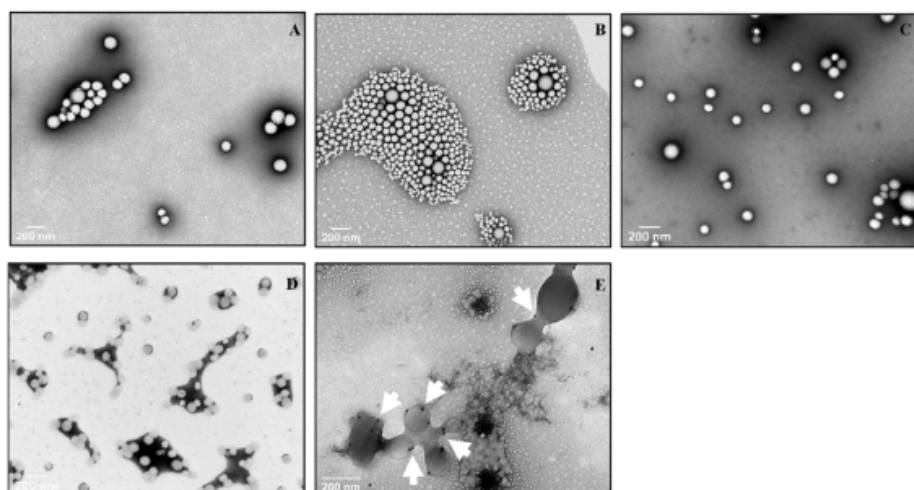


Fig. 1. Image of PLGA (A), PLGA-PEG (B), B72.3 Functionalized PLGA-PEG (C) and IgG Functionalized PLGA-PEG (D) nanoparticles obtained by Transmission Electron Microscopy (TEM). (E) Image resulting from ImmuoTEM with B72.3 Functionalized PLGA-PEG nanoparticles. The white arrow represents the antibody bound to the nanoparticle. Scale bar: 200 nm.

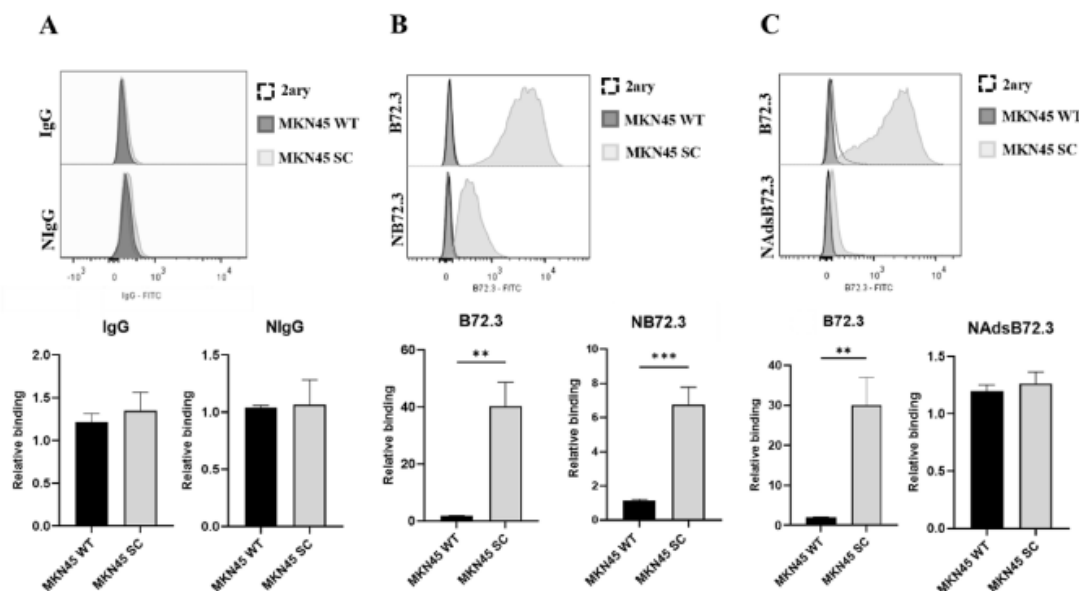


Fig. 2. Flow cytometry analysis of MKN45 WT and MKN45 SC, which were incubated with (A) IgG Functionalized PLGA-PEG NP (NlgG), (B) B72.3 Functionalized PLGA-PEG NP (NB72.3) and (C) B72.3 Adsorbed PLGA-PEG NP (NAdsB72.3) nanoparticles, to evaluate the targeting ability and specificity of the nanoparticles under static fluid conditions. Data presented as representative histograms of 3 independent experiments and mean of relative binding $\pm$ SD.

3.1.1. Conjugation efficiency of antibodies to the nanoparticles

The surface of PLGA-PEG-COOH NPs (NPP) was functionalized with one of the two antibodies: the monoclonal antibody B72.3 or a control mouse IgG. As mentioned before, B72.3 antibody is directed to the detection of the STn antigen, the tumor-associated truncated glycan [18, 21] expressed in MKN45 SC cell line, and absent in the control MKN45 WT cell line [37]. A NP functionalized with an unspecific IgG1 antibody was also developed to be used as a non-targeted control NP, NlgG. Our results showed that the main difference between functionalized and non-functionalized NPs is the PDI (Table 1). Besides the values being lower than 0.2, the functionalized NPs have higher values of PDI, showing that these NPs are more heterogeneous and not so well distributed. This difference might be due to aggregation of NPs which may result from sample processing, during the functionalization protocol, using the carbodiimide process [42]. Nevertheless, by being vortexed the NPs were resuspended again. In order to confirm the efficacy of NPs functionalization and quantify the conjugation efficiency (CE), an ELISA assay was performed. This indirect CE quantification was performed using the washes from functionalization method to quantify the antibody that did not bind. It was shown the presence of antibodies with CE of about 75% for IgG1 (from 33 μ g) and around 85% in the case of B72.3 (from 33 μ g), representing successful functionalization of NPs with both antibodies (Table 1). Previous studies report that low CE might be associated with lower amounts of antibody [34,43,44]. The high CE percentage observed in the developed NPs can be explained by the use of a high amount of antibody to functionalize these NPs, increasing the linkages between antibody and the NPs. To further confirm the CE, an ImmunoTEM characterization was performed showing the efficient binding of the antibody B72.3 to the nanoparticle as indicated by the black dot in the micrograph (Fig. 1E).

3.2. Characterization of lapatinib-loaded nanoparticles

The capacity of these NPs to encapsulate a Tyrosine Kinase Inhibitor

(TKI) molecule with success was also evaluated and characterized. During the last two decades, several molecules targeting Receptor Tyrosine kinases (RTKs) were used as therapy in different types of cancer. TKIs are known as inhibitory drugs that can block the growth and spread of tumor cells by interfering with key molecules (RTK) involved in malignancy [45,46]. However, their effectiveness is limited by the acquired resistance or adverse effects like toxicity/side effects. These adverse effects can be avoided by TKI encapsulation in a nanosystem and deliver in a controlled manner [47–49]. Regarding Lapatinib, a dual tyrosine kinase inhibitor (HER2 and EGFR [24]), its clinical use is done in a restrictive form and is characterized by extensive albumin binding capacity, poor oral bioavailability, and poor water solubility, which results in poor and incomplete absorption from the gastrointestinal tract. Lapatinib is commercialized for oral intake only [50,51]. Therefore, it becomes imperative to formulate alternate efficient nano-delivery systems for administration through non-oral routes, for active targeting, and to scale-up by pharmaceutical scientists followed by their clinical trials by clinical experts. To overcome this obstacle, we developed a stable and high-loaded polymeric formulation encapsulating Lapatinib with the surface functionalized with mAbs against STn antigen. The physico-chemical characterization can be assessed on Table A.1, as the association efficacy, drug loading and CE. The CE of antibodies was calculated by LC-MS and was made by a direct method of quantification, as we used the sample of NPs and after degraded enzymatically the antibody we proceeded to the analysis. The values of CE for B72.3 and IgG are ~65% (from 33 μ g) for each. The values showed to be lower comparing to empty NPs, but we hypothesized that this can be due to the fact that the drug could limit the places to bind the antibody. Further, we also demonstrated the successful encapsulation, with an association of efficacy up to 50% of 1 mg of Lapatinib and a drug loading capacity of about 2.2%, quantified by a sensitive methodology, LC-MS, resulting in 20,467 mg of Lapatinib-loaded PLGA-PEG-COOH NPs per batch. On the literature, there are some reports that show higher levels for the percentage of encapsulation of Lapatinib. For example, Buss et al., reports an

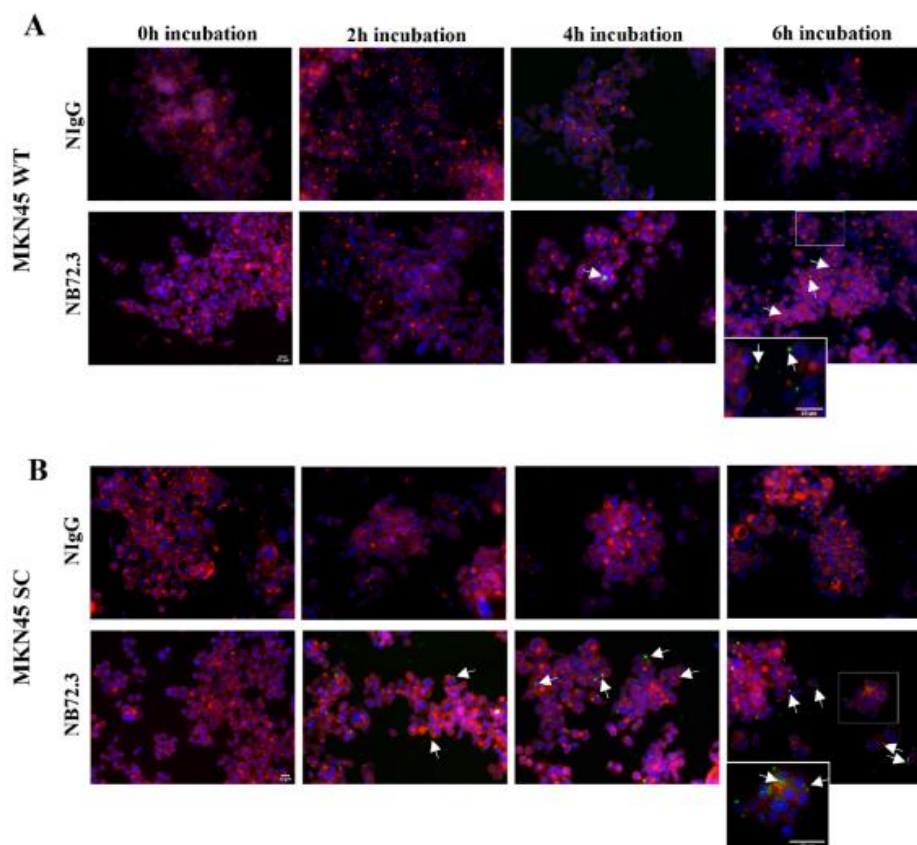


Fig. 3. Immunofluorescent staining of a panel of gastric cancer cells (MKN45 WT (A) and MKN45 SC (B)) incubated with NlgG and NB72.3 nanoparticles during different timepoints at 37 °C to evaluate the interaction between cell-nanoparticles under static fluid conditions. Nuclei are stained in blue, cytoskeleton (phalloidin) is stained in red and the antibody conjugated with the NPs are observed in green, the white arrow indicates the nanoparticles agglomerates. Images were acquired with magnification 20 $\times$ for all timepoints and with magnification 63 $\times$ for timepoint 6h. Scale bar: 20 μ m.

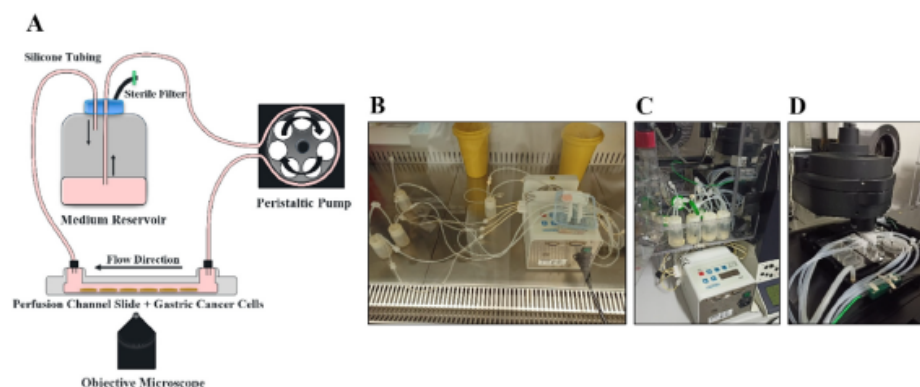
association efficacy of 100% with a theoretical mass of 0.250 mg [52]. Taking this into consideration we can conclude that in our NPs we were able to encapsulate higher amounts of drug, 0.467 mg of Lapatinib. Lastly, we looked at the morphology of the NPs by ImmunoTEM which showed the presence of B72.3 on NP surface and the spherical shape of the NPs, indicating that these developed NPs are a good model to be applied in the future for drug delivery studies (Figure A.1).

Further, we performed a functional assay *in vitro* to evaluate the effect of the drug on the cancer cells with HER2 and EGFR amplification. We have used resazurin viability assay in GC cells expressing Sialyl-Tn (MKN45 SC) upon incubation with 1 μ g/mL of Lapatinib, NlgG and NLB72.3 during 24h and 48h. Our results showed that NLB72.3 induces higher specific effect, decreasing the metabolic activity in GC cells expressing STn at 24h and 48h of incubation (Figure A.2). Regarding the effect of NlgG, this may stem from NPs sedimentation at static conditions and consequent unspecific release of Lapatinib.

3.3. Interaction of gastric cancer cells and nanoparticles under static fluidic conditions

In order to evaluate the capacity of the NPs functionalized with the

antibody against the STn glycan to target tumor cells of interest we evaluated the interaction between gastric cancer cells with NPs under static fluid conditions followed by detection through flow cytometry. Empty B72.3 Functionalized PLGA-PEG NPs (NB72.3) and IgG Functionalized PLGA-PEG NPs (NlgG) (100 μ g/mL) were incubated with MKN45 SC cells expressing STn and MKN45 WT cell line without expression of STn antigen. As seen in Fig. 2A, cell association was absent for non-targeted control, the NlgG, in both cell lines. On the contrary, a clear cell association was observed for NB72.3 with the STn expressing cell MKN45 SC, whereas no interaction was observed with MKN45 WT (Fig. 2B). The higher association and uptake of NB72.3 to MKN45 SC comparing to NlgG, shows the efficient target ability and specificity of these NPs. In other hand, the lack cell association was observed between NlgG and both cell lines, supporting that the NP recognition observed is specific (Fig. 2A). In fact, previous studies have shown that NPs targeting glycosylation have the potential to increase the specificity and therefore the safety and efficacy of carried therapeutic antitumor agents delivery [7,12]. Our results confirmed the capacity of recognition and specificity of the developed NPs targeting the cancer-associated STn glycan antigen. We also validated the capacity of recognition of our NPs in two other gastric cancer models: AGS WT and AGS SC (Figure A.3.A-B), without



Scheme 2. (A) Schematic representation of methodology behind the development of studies under flow. (B) Mounting the microfluidic system in sterile conditions. (C) Widefield microscope with the peristaltic pump connected to the microfluidic system. (D) Perfusion channel slide connected by silicone tubing to microfluidic system and prepared to live acquisition by widefield microscope.

and with STn antigen expression, respectively. We demonstrated that NB72.3 and NiGg have a similar behavior with these two cell models comparing to MKN45 WT and MKN45 SC. We showed once more that they have efficient target ability and specificity.

Further evaluation of the specificity of the antibody covalently linked to the NP surface, was performed using a control experiment with B72.3 Adsorbed PLGA-PEG NPs (NAdsB72.3). We incubated B72.3 antibody with NPs in the absence of EDC and NHS and therefore without the activation of the carboxylic group to react to the amine group of the antibody. Nanoparticles NAdsB72.3 showed lower capacity of binding to cells expressing STn (MKN45 SC) (Fig. 2C) demonstrating that these NPs are not able to detect the STn expressing cells, and indeed requires the NP with the covalently linked antibody NB72.3. Overall, our data demonstrate the specificity of the conjugation method and highlights the importance of the NP displaying the covalently linked antibody. Our results are in agreement with a previous study with PLGA-NPs functionalized with aptamers in the presence and absence of EDC and NHS, that demonstrates the specificity of their conjugation reaction [53]. We also wanted to demonstrate that when we treat MKN45 SC with the neuraminidase enzyme to remove sialic acid groups, our NPs functionalized with antibody against STn antigen, are still showing specificity. Our results confirm the specificity of NB72.3 and the increase of Tn antigen upon treatment with neuraminidase enzyme, demonstrating that the treatment was efficient (Figure A.3. C).

To gain insight into the cellular and intracellular localization of the NPs, we examined the targeting ability and specificity of our polymeric NPs by immunofluorescence. The different cell models were incubated during different timepoints (0, 2, 4 and 6h) with functionalized NPs (100 $\mu\text{g/mL}$) in static fluid conditions (Fig. 3). Aggregates of NB72.3 were observed to have a much higher cell affinity to MKN45 SC, when comparing with the NiGg, which is practically undetectable the binding to MKN45 SC (Fig. 3B, arrow). In fact, NiGg behaved exactly as a non-targeted NP showing no binding to both cell models. Additionally, the association of NB72.3 to MKN45 SC showed to be time-dependent, as an increase in the binding of particles over time was observed. However, accumulation of aggregates of NB72.3 were also found close to the MKN45 WT cells, particularly at incubations of $\geq 4\text{h}$ (Fig. 3A, arrow). This observation may stem from the static fluid conditions experiments. Previous studies have reported that in static conditions, NPs tend to be accumulated and deposited close to or on top of the monolayer cells [28, 30]. Such static condition can promote the unspecific interaction between the cells and NPs. On the other hand, in both experiments, we cannot exclude a limitation of the carbodiimide chemistry method,

where the lack of control over the antibody orientation into the NP surface poses a primary disadvantage of this method and should be taken into consideration. The coupling between groups and crosslinkers is not selective and controlled, so if the antibody does not have the binding sites available due to the orientation into the NP, this will reduce the targeting opportunity [54]. Moreover, assuming that the binding sites for detection by the secondary antibody- HRP are not available, the signal detected will not be significant, although this not exclude the presence of the antibody on the NPs surface [54,55].

3.4. Interaction of gastric cancer cells and nanoparticles under flow fluidic conditions

The major purpose behind developing NPs for drug delivery applications is the optimization of parameters that influence cell-NPs interactions. A common approach is to perform validation of NPs using *in vivo* animal experiments. Our results under static conditions highlighted some important issues related to the sedimentation of NPs, resulting in their heterogeneous dispersion with the target cells, and accumulation of toxic waste products [28,30,56]. Therefore, a mechanical stress methodology for evaluating NP risk management, dynamic under flow conditions, drug delivery and avoiding extensive animal validations is needed. We evaluated the effect of dynamic culture conditions in several conditions. By applying such studies in the absence of NPs we could adjust several variables, such as the medium supplementation, the shear stress, and allowed to define a proper coating condition to maintain cell culture and set light exposure to avoid phototoxicity during imaging. Such conditions were defined until no significant difference in morphology was found under static or dynamic conditions for both cells expressing or not our target of interest.

Furthermore, we also studied the effect of shear stress (2dyn/cm²), generated by fluid flow through the channels, on adhesion of NPs to MKN45 SC and MKN45 WT cells. To do so, cells were incubated with different empty NPs labelled with FITC groups, functionalized and non-functionalized (100 $\mu\text{g/mL}$), and live cell imaging was performed using over 5,5h (Scheme 2). We have used a theoretical mass of 5% PLGA-FITC and since the FITC conjugation is covalent, early release of the fluorophore from NPs is not expected. Our results showed that the targeting ability and specificity of the NPs is maintained under flow conditions as aggregates of B72.3 Functionalized PLGA-FITC-PEG NPs (NF-B72.3) binding only to the MKN45 SC cell model were observed (Fig. 4B, arrows; Vid. A.1–6). Although the control IgG Functionalized PLGA-FITC-PEG NPs (NF-IgG) did not bind to any cell model, we were able to see that

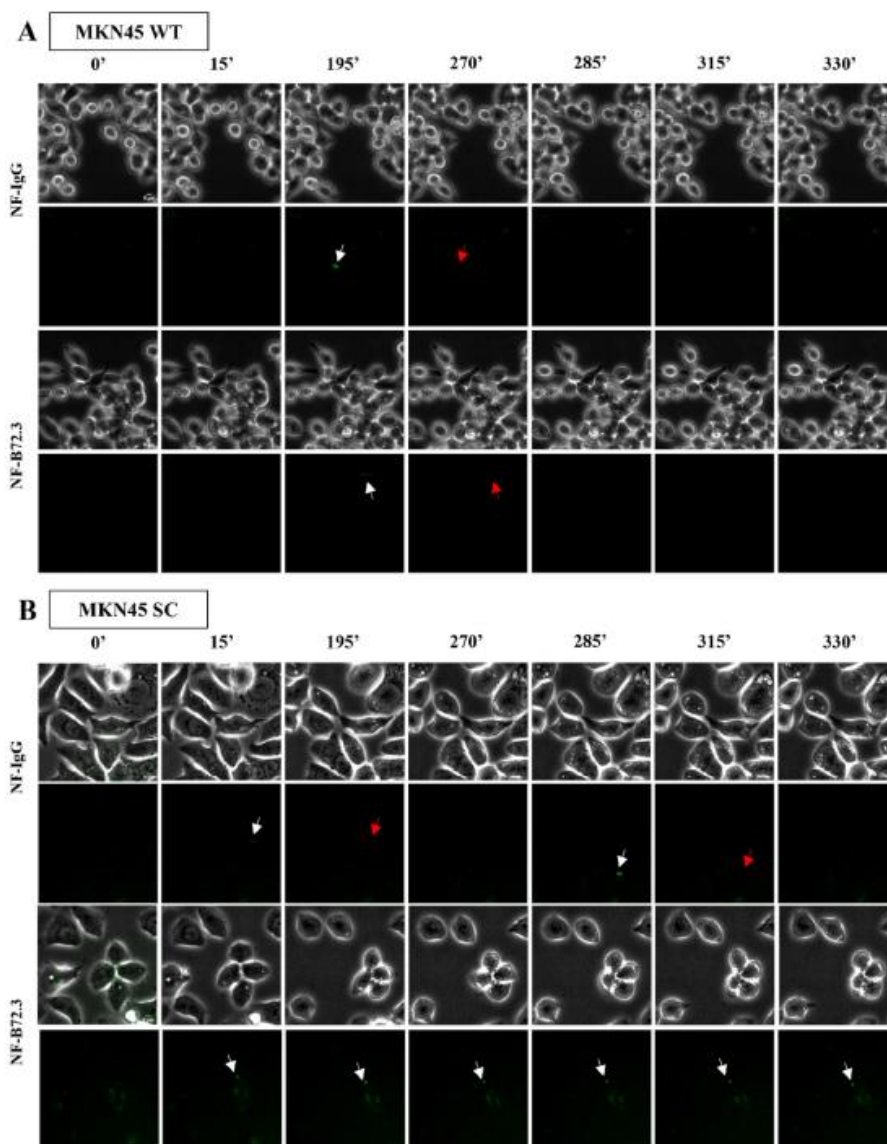


Fig. 4. Association of NF1gG and NFB72.3 to (A) MKN45 WT and (B) MKN45 SC after 5.5h incubation under live flow conditions (shear stress of 2dyn/cm²) at 37 °C. Representative images obtained with a widefield microscope at magnification 40×. Nanoparticles can be observed in green, the white arrow indicates the nanoparticles agglomerates, and the red arrow indicates when the nanoparticles disappear. The same brightness and contrast settings were used for the FITC (green) channel in all images. Scale bar: 5 μm.

both NPs labelled with FITC and functionalized appear to touch the cells in a non-specific way (Fig. 4, A-B, arrows; Vid. A.1–6). Nevertheless, the lack of adhesion of control NPs to cells further illustrates the specific targeting of this nanosystem to STn expressing cells. In contrast with the results observed under static conditions, we were able to avoid the unspecific accumulation of NPs aggregates under flow conditions, supporting the efficacy of this experiment for evaluation of the NPs interaction with the cells.

On the other hand, our results showed that cell morphology changes with several hours of live image acquisition exposure, suggesting a potential limitation of the current model of NP testing under flow conditions related to the maximum time of acquisition of images. These observations are most probably due to the shear stress, since the cells started to show signs of stress when following them over a very long period (more than 7h) (data not shown). Another reason that can support this observation, is the fact that contrarily to other studies, we performed

the live acquisition outside of an incubator. The proposed flow system is a novelty in the field as it allows to follow over time the interaction of NPs with the cells in live image acquisition. Most of the studies published so far rely on the fixation of the cells after a certain incubation period with the NPs under flow conditions, around 24h or 36h [26,27,32,57,58]. However, by doing so, we cannot infer about the interaction between them. In the future, this microfluidic system can be applied to the study of other nanoparticle-endothelial cell interactions, which can guide the engineering of NPs for *in vivo* medical applications. As example, shear stress-activated processes, in circulation, are reported to significantly affect the NP internalization by endothelial cells [26,27,57]. Therefore, several targeting strategies are employed to ensure the delivery of a sufficient payload of drug to the vascular regions under physiologic shear stress conditions, whereby the most promising experimental results have thus far been reported with endothelial adhesion molecule-targeting approaches [26,27,57,58].

Finally, we demonstrate that none of the compounds of the developed NPs (polymers or monoclonal antibodies) have a cytotoxic effect in our cell models during 5,5h and 24h, ensuring that our NPs do not affect the cells' metabolic activity (Figure A.4).

4. Conclusions

Our results showed that polymeric NPs functionalized with B72.3 antibody can target STn glycan, highly expressed by cancer cells. This is the first time that NPs functionalized with an anti-STn antibody were used for targeting STn expressing-cancer cells. Additionally, we evaluated the targeting capacity of these NPs in live cancer cells and under flow conditions. The live tracking of NPs is an innovative platform for *in vitro* experiments since it simulates the physiological flow which may mimics the behavior and interaction of cells and NPs in these conditions. Therefore, we propose that the live tracking using a microfluidic approach is a robust method to evaluate and characterize the targeting capacity the NPs and the effects they can have on the targeting cells. Finally, the specificity of these novel NPs demonstrates a great potential to be translated to *in vivo* studies of drug delivery.

Credit author statement

FD, JG, BS and CAR conceptualize the manuscript. FD and FS developed the nanoparticles and their functionalization. HO performed the mass spectrometry experiments. DC and FD developed the ELISA method. FD, MA and PS developed under flow experiments. FD prepared the schemes and figures. MA prepared the videos from under flow experiment. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtbio.2022.100417>.

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Supplementary Files

Supplemental Files (SF) for Publication

Table A.1: Properties of functionalized and non-functionalized nanoparticles loaded with Lapatinib, including the mean size, polydispersity index (Pdl), surface charge, association efficacy (AE), drug loading (DL) and conjugation efficacy (CE). The values are represented as mean values $\pm$ SD (N=3). Non applicable (n.a.)

	Size (nm)	Pdl	Surface Charge (mV)	AE (%)	DL (%)	CE (%) LC-MS
Lapatinib-loaded PLGA-PEG NP	124.8 $\pm$ 1.8	0.153 $\pm$ 0.220	-5.1 $\pm$ 0.8	46.7 $\pm$ 4.2	2.22 $\pm$ 0.20	n.a.
B72.3 Functionalized Lapatinib-loaded PLGA-PEG NP (NLB72.3)	237.4 $\pm$ 6.2	0.267 $\pm$ 0.074	-13.9 $\pm$ 2.3	24.7 $\pm$ 4.3	1.17 $\pm$ 0.20	67.1 $\pm$ 9.5
IgG Functionalized Lapatinib-loaded PLGA-PEG NP (NLIgG)	204.3 $\pm$ 5.2	0.243 $\pm$ 0.026	-12.0 $\pm$ 1.9	25.7 $\pm$ 3.1	1.22 $\pm$ 0.15	66.0 $\pm$ 6.9

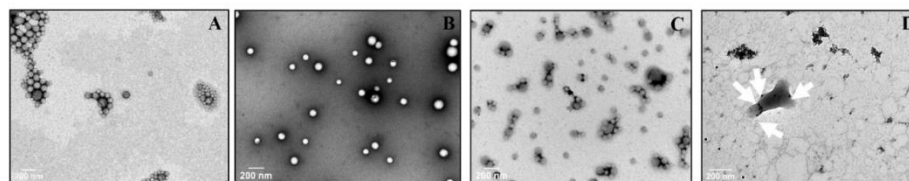


Figure A.1: Representation of Lapatinib-loaded PLGA-PEG (A), B72.3 Functionalized Lapatinib-loaded PLGA-PEG (B) and IgG Functionalized Lapatinib-loaded PLGA-PEG (C) nanoparticles obtained by Transmission Electron Microscopy (TEM). (D) Image resulting of ImunoTEM with B72.3 Functionalized Lapatinib-loaded PLGA-PEG nanoparticles. The white arrow represents the antibody bound to the nanoparticle. Scale bar: 200 nm.

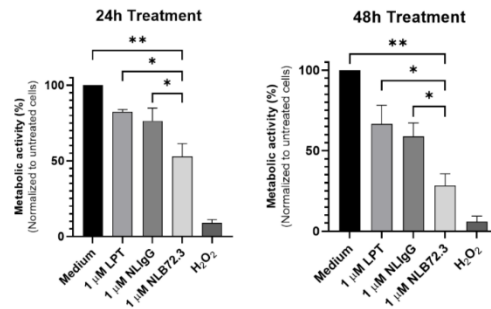


Figure A2: Metabolic activity of MKN45 SC cell model after treatment during 24h and 48h with 1 μ m/ml of free Lapatinib (LPT), IgG Functionalized Lapatinib-loaded PLGA-PEG-COOH NPs (NLIgG) and B72.3 Functionalized Lapatinib-loaded PLGA-PEG-COOH NPs (NLB72.3), measured by resazurin assay. Hydrogen peroxidase (H₂O₂) was used as positive control. Data presented as representative histograms of 2 independent experiments and mean of relative binding $\pm$ SD.

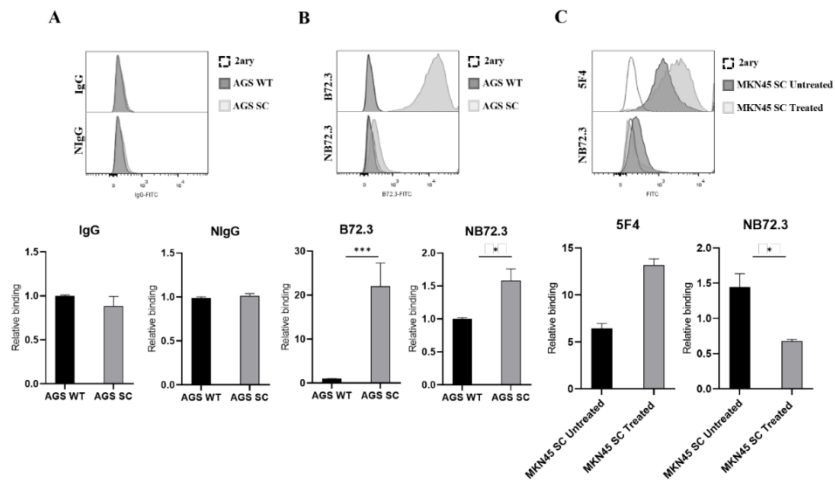


Figure A.3: Flow cytometry analysis of AGS WT and AGS SC, which were incubated with (A) IgG Functionalized PLGA-PEG NP (NlgG) and (B) B72.3 Functionalized PLGA-PEG NP (NB72.3) nanoparticles, to evaluate the targeting ability and specificity of the nanoparticles under static fluid conditions. (C) Flow cytometry analysis of MKN45 SC treated and untreated with neuraminidase enzyme, which were incubated with antibody against Tn antigen (5F4) and B72.3 Functionalized PLGA-PEG NP (NB72.3) nanoparticles, to evaluate the targeting ability and specificity of the nanoparticles. Data presented as representative histograms of 3 independent experiments and mean of relative binding $\pm$ SD.

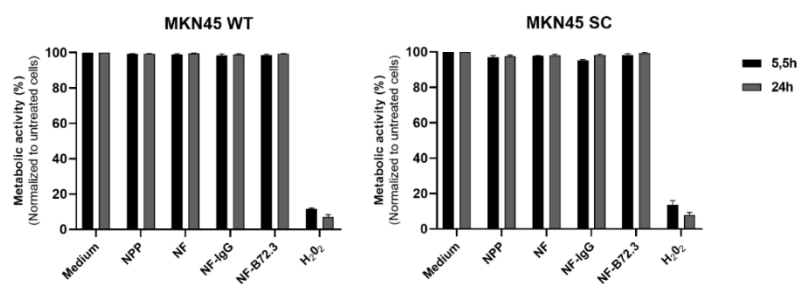


Figure A.4: Metabolic activity following treatment during 5,5h and 24h with 100µm/ml of PLGA-PEG-COOH NPs (NPP), FITC-labelled NP (NF), IgG Functionalized PLGA-FITC-PEG NP (NF-IgG) and B72.3 Functionalized PLGA-FITC-PEG NP (NF-B72.3), measured by resazurin reduction assay in MKN45 WT and MKN45 SC. Hydrogen peroxidase (H₂O₂) was used as positive control and untreated cells as negative control. Data presented as representative histograms of 2 independent experiments and mean of percentage of metabolic activity +/- SD.

CHAPTER 3

Paper II

Nanoparticles targeting Sialyl-Tn for efficient tyrosine kinase inhibitor delivery in gastric cancer

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Manuscript Under Revision

Nanoparticles targeting Sialyl-Tn for efficient tyrosine kinase inhibitor delivery in gastric cancer

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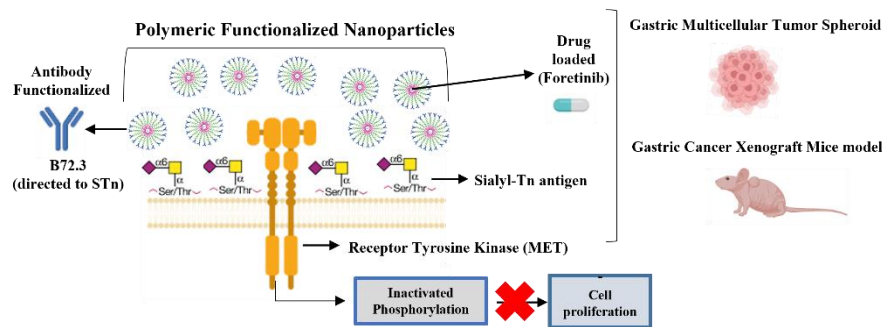
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Keywords: Polymeric Nanoparticles; Sialyl-Tn antigen; Receptor Tyrosine Kinase inhibitor; Foretinib; Cancer therapy

ABSTRACT

Gastric cancer is the fourth leading cause of cancer-related deaths worldwide and, therefore, it is urgent to develop new and more efficient therapeutic approaches. Foretinib (FRT) is an oral multikinase inhibitor targeting MET and RON receptors tyrosine kinases (RTK) that has been used in clinical trials for several solid tumors. Targeted uptake of therapeutic polymeric nanoparticles (NPs) represents a powerful approach to cancer cell drug delivery. We had previously developed a nanodelivery system composed of polymeric NPs functionalized with B72.3 antibody targeting Sialyl-Tn antigen, a tumor-associated glycan. Herein, these NPs were loaded with FRT and their capacity to deliver the drug in multicellular tumors spheroids (MCTS) and in mouse models was evaluated. Our data indicated that B72.3 functionalized FRT-loaded PLGA-PEG-COOH NPs (NFB72.3) specifically target gastric cancer MCTS expressing the STn glycan (MKN45 SimpleCell (SC)), leading to a decrease of activation of phospho RTK and reduced cell viability. *In vivo* evaluation using MKN45 SC xenograft mice revealed that NFB72.3 were able to decrease tumor growth, reduce cell proliferation and tumor necrosis area. NFB72.3-treated tumors also showed inactivation of phospho-MET and RON. This study demonstrates the value of using NPs targeting STn for FRT delivery, highlighting its potential as a therapeutic application in gastric cancer.



1. Introduction

Gastric cancer (GC) represents one of the tumors with highest incidence and mortality rate worldwide. Most of the cases are diagnosed at advanced stage and the therapeutic approaches are currently very limited [1, 2]. Major targets for cancer therapy are receptors tyrosine kinase (RTKs). Currently, Trastuzumab (Herceptin) is a FDA approved monoclonal antibody used in the clinics to target human epidermal growth factor receptor 2 (HER2) in metastatic HER2-overexpressing tumors [3, 4]. However, this targeted therapy has limited use and there is a high pressure to find new therapeutic options in GC. In addition, small-molecule tyrosine kinase inhibitors (TKI) that target other RTKs expressed in the tumors, represent a potential application for GC treatment.

RON is a RTK activated by a macrophage stimulating protein growth factor that plays a role in tumorigenesis and poor prognosis. In GC, RON is described to be expressed in about 50% of GC tissues [5]. Hepatocyte growth factor (HGF) receptor, also known as MET is also a RTK amplified in approximately 20% of the diffuse subtype of GC [6]. When aberrantly activated, MET is a key driver of tumor cell growth, motility, invasion, metastasis and angiogenesis [7, 8]. Foretinib (FRT) is a small-molecule tyrosine kinase inhibitor (TKI), that inhibits MET phosphorylation, migration and invasion of MET-amplified human GC cell lines [9]. Studies *in vivo* reported that FRT retarded tumor growth through MET receptor [10-14]. In addition, FRT was also reported as having activity, although less potent than MET, against RON, Tie-2, PDGFR- β , AXL and VEGFR [9, 12, 15, 16].

In clinical trials, FRT has been evaluated for its antitumor potential in several cancers [17-21]. In Phase I studies, FRT inhibited activation of MET and decreased the proliferation of solid tumors in patients [17, 22]. In GC Phase II clinical trial with FRT, the drug failed to meet its primary endpoint of an objective response rate >25% [18]. Despite evidence of FRT effect in decreasing MET activation, FRT lacked antitumor

efficacy in unselected patients with metastatic GC by demonstrating no tumor regression. Even so, this study was applied on a small cohort of patients, where only less than 5% of the subjects exhibited MET amplification [18].

The combination of targeted therapies and controlled drug release are attractive approaches for decrease drug cytotoxic effects and improve efficient specific delivery of the drug to the cancer cells [23]. Nanoparticles (NPs) are known as a leading system to enhance drug delivery in the treatment of multiple diseases, including cancer [24]. One of the most notable properties of the therapeutic NPs is their capacity to efficiently encapsulate drugs with poor solubility [23, 24]. However, development of efficient, targeted and safe drug delivery approaches remains challenging due to limited known cancer-specific targeting antigens [23-25].

In cancer, a wide variety of molecules have been assessed as potential targets, such as the glycan antigens [26-28]. Aberrant glycosylation affects several pathological steps of the tumor development and progression [29, 30]. Functionally, glycans actively control cancer cell signaling, tumor cell dissociation and invasion, cell-matrix interactions, angiogenesis, metastasis and immune modulation [29]. The sialyl-Tn antigen (STn) is a truncated O-glycan which consists of a sialic acid α -2,6 linked to GalNAc α -O-Serine/Threonine (Ser/Thr). The expression of the sialyltransferase ST6GalNAc1 as well as mutations in or loss of heterozygosity of the COSMC gene has been associated with its biosynthesis [31-33]. STn is expressed by more than 80% of human carcinomas, including GC, and rarely expressed in normal tissues [31, 32, 34]. This sialylated glycan is associated with malignancy and poor prognosis in cancer patients. It is reported that expression of the tumor-associated STn-antigen reduces cell adhesion in prostate cancer and increases migration and invasion in breast and gastric cancer cells [34, 35]. These features point out STn antigen as a potential target for therapeutic applications in cancer. In this study we propose a nanodelivery system based on poly(lactic-co-glycolic acid) (PLGA) NPs loaded with FRT for drug delivery. These NPs, were functionalized with a monoclonal antibody (mAb) directed to STn antigen [36]. PLGA is a commonly biocompatible and biodegradable polymer used to produce polymeric NPs that was already approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) for medical applications [37-39]. One of the advantages of PLGA polymer is its capacity to be chemically modified, which enhances NPs ability for an active targeting [40, 41].

We have previously developed NPs formulated with PLGA and pegylated PLGA with a terminal carboxylic acid functional group (COOH) functionalized with B72.3 mAb that recognizes STn antigen. We have shown that this nanosystem specifically targeted GC cells expressing STn antigen, in both static and fluidic conditions. No binding or uptake was detected in GC cells not expressing the STn antigen [36].

In the present study, we evaluated the suppressive effects of NPs loaded with FRT and functionalized with an antibody against STn antigen on GC cells both *in vitro* and *in vivo*. More specifically, its effect on proliferation, apoptosis and the RTKs activation. We characterized the ability of our nanosystem to interact specifically with GC cells expressing STn, together with its antiproliferative and cytotoxic effect in multicellular tumor spheroids (MCTS). We also evaluated for the first time *in vivo*, the capacity of these NPs upon intravenous administration in reducing tumor growth in mice bearing xenografts of GC cells expressing STn antigen and MET receptor. This proof-of-concept study highlights the potential of this novel nanodelivery approach directed to STn as a promising strategy for GC treatment.

2. Materials and methods

2.1 Nanoparticles development

2.1.1. Nanoparticles production

The Foretinib-loaded PLGA-PEG-COOH NPs were prepared using nanoprecipitation method as previously described by us [36, 42]. Briefly, 19 mg of Poly(lactide-co-glycolide) (PLGA) 5004A (provided by Corbion) and 1 mg of Poly(lactide-co-glycolide)-b-poly(ethylene glycol)-carboxylic acid endcap (PLGA-PEG-COOH) (PolySCiTech) were added to 1 mg of FRT (LogP value: 5.5) (SantaCruz, sc-364492) (dissolved in DMSO, 40µl) and left in 1,960mL of acetone (ThermoFisher) to allow the complete dissolution. Further, this organic solution was transferred using a 25G needle to an aqueous solution of 0,5% (w/v) Pluronic F-127 (Sigma-Aldrich) (20mL) under magnetic stirring (300rpm) for 3h and the remaining organic solvent was removed by evaporation. Finally, NPs were washed with MiliQ water, using 100 kDa Amicon® filters, centrifuged for 20min at 2,000g, thrice, and resuspended in 1,5mL of phosphate-buffered saline (PBS). The same nanoprecipitation method was also used to produce Foretinib-loaded PLGA and empty NPs without the presence of PLGA-PEG-COOH and FRT, respectively.

2.1.2. Nanoparticles-antibody conjugation

Monoclonal antibody (mAb) B72.3 (anti-STn)[43] was purified from hybridoma culture media using the HiTrap™ Protein G HP 1mL columns of purification (Sigma-Aldrich). The conjugation of NPs with anti-STn antibody (B72.3) or IgG1 Isotype Control Purified Mouse Monoclonal IgG antibody (R&D Systems) was performed via carbodiimide chemistry as previously described by us [36].

2.2 Characterization of nanoparticles

2.2.1 Physicochemical characterization of nanoparticles

All formulations of NPs, diluted in sodium chloride (NaCl) 10 mM, pH 7.4, were physiochemically characterized regarding the particle size (nm), polydispersity index and surface charge (ζ -potential in mV) using Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd) as previously detailed [36].

2.2.2 Association efficacy, drug loading and conjugation efficiency of nanoparticles

2.2.2.1 LC-MS

The association efficacy and drug loading of Foretinib loaded in NPs was determined by Liquid chromatography–mass spectrometry (LC-MS). NPs formulations were exposed overnight at freeze dryer and then were resuspend in dimethylformamide for drug extraction. The LC-MS analysis was done in an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) as previously demonstrated by Diniz et al [36].

Association Efficacy (%) = (Mass of the drug in NPs / Initial Mass of drug in the NPs) x 100

Drug loading capacity (%) = (Mass of the drug in NPs / Initial Mass of the NPs) x 100

We also performed LC-MS to confirm the conjugation efficiency of B72.3 or IgG antibody to NPs, conjugated with Foretinib, by direct method as previously described[36].

Conjugation Efficiency (%) = (Mass of antibody in NPs / Initial Mass of antibody in the NPs) x 100

2.2.2.2 Enzyme-linked immunosorbent assay (ELISA)

The binding efficiency of B72.3 antibody and IgG1 control to NPs was also quantified by indirect method using enzyme-linked immunosorbent assay (ELISA) as previously described [36]. All samples were done in triplicate. The obtained absorbance values were used to calculate the conjugation efficiency (%).

Conjugation Efficiency (%) = ((Absorbance of the input – Absorbance of the sample) / Absorbance of the input) x 100

2.2.3 Morphology of nanoparticles

The morphology of the NPs was observed through Transmission Electron Microscopy (TEM). Briefly, 10 μ L of each NPs sample were mounted on nickel grids and left to stain for 2 min approximately. Afterwards, the excess liquid was removed with filter paper, and a Uranyl Acetate solution (1%) was added and left to incubate for 10s. Samples were viewed on a JEOL JEM 1400 TEM (JEOL, Tokyo, Japan) operated at 80kV, and images were digitally recorded using a CCD digital camera Orius 1100W (Tokyo, Japan).

2.3 Cell lines and culture conditions

Gastric carcinoma cell line MKN45 (MKN45 WT) was obtained from the Japanese Cancer Research Bank (Tsukuba, Japan). The MKN45 SimpleCell model (MKN45 SC) was generated by targeting the C1GALT1 (COSMC) gene using zinc-finger nuclease precise gene editing, which results in knockout COSMC, as previously described [44]. Cells were grown as monolayer in cell culture flasks, maintained at 37°C in an atmosphere of 5% CO₂, in RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum (FBS) (Biowest, USA).

2.4 Generation of gastric multicellular tumor spheroids

Gastric multicellular tumor spheroids (MCTS) formation was conducted by two different approaches according to the experimental functional protocol:

Cell suspensions of 250 cells/microwell were seeded in ULA plates (Corning, New York, NY, USA) according to the protocol described in [45, 46] and in 3D Petri Dish® (MICROTISSUES® technology, MicroTissues Inc., Sigma-Aldrich, St. Louis, MO, USA) in [45, 46]. Briefly, 12-series agarose micro-molds were prepared using 2% agarose in 0.9% of NaCl. Micro-molds were placed in 12-well plates and equilibrated with RPMI 10% FBS before cell seeding.

A 30 min incubation period was used for cells to settle into the micro-molds and additional medium was finally added to the wells. During the following 5 days, spheroids formation both in ULA plates and 3D Petri Dish®, were monitored using an inverted Leica DMi1 microscope for cell culture (Leica Microsystems, Wetzlar, Germany).

2.5 Flow cytometry analysis

The bind specificity and affinity of the NPs were evaluated by flow cytometry as previously described [36]. MCTS from MKN45 WT and MKN45 SC cells were seeded in 3D Petri Dish 5 days before analysis. Briefly, MCTS were destroyed using Accutase cell detachment solution (Grisp), and 1 million cells were used in each condition. Cells suspension was fixed with 2% Paraformaldehyde (PFA) (Alfa Aesar) for 20 min at room temperature (RT), and then washed twice with PBS at 350g for 5 min. Cells were permeabilized with 0,1% Triton, PBS for 5min at 4°C and washed twice with PBS. Afterwards, cells were incubated under gentle agitation at RT with functionalized NPs (100ug/mL), B72.3 antibody (33ug) or IgG1 antibody (33ug) for 2 h. After, cells were washed and incubated with the secondary antibody goat anti-mouse Alexa Fluor® 488 (1:200) (Invitrogen) for 30 min in the dark. Cells were washed and resuspended in PBS, strained and labeled with DAPI (2ug/mL) (Sigma-Aldrich). Cells suspension was analyzed using BD FACS Canto™ II (BD Biosciences, San Jose, CA, USA). Three independent experiments were conducted. Data were analyzed using FlowJo (BD Biosciences, San Jose, CA, USA).

2.6 Protein analysis by Western Blot

After 5 days of MCTS formation in molds, cells were treated with the FRT diluted in cell culture medium serum-free at concentrations ranging from 0.01 to 5 μ M for 24 h. Further, we tested a range of concentrations of NFB72.3 between 0.5 and 10 μ M for 24 h. Following that, we treated MCTS with 1 μ M of free FRT, 1 μ M of NF IgG and 1 μ M of NFB72.3 during 3 timepoints: 6, 24 and 36h. Control conditions containing the corresponding amount of the vehicle (DMSO) were performed. Total protein lysates were collected using lysis buffer 17 (R&D Systems) supplemented with PhosSTOP phosphatase inhibitor cocktail (Sigma-Aldrich) and cOmplete™ protease inhibitor cocktail (Sigma-Aldrich). Protein concentration of each condition was determined using the DC protein assay (BioRad). Same amounts of total protein lysates were electrophoresed under reducing conditions on polyacrylamide gels and transferred onto

nitrocellulose membranes (GE Healthcare Life Sciences). After 1 h blocking at RT, membranes were incubated overnight at 4 °C with primary antibodies: MET (D1C2, Cell Signaling Technology, dilution 1/2000), RON (E-3, Santa Cruz Biotechnology, dilution 1/100), p-MET (Y1234/1235, D26 clone, Cell Signaling Technology, dilution 1/2000), p-RON (Y1238/Y1239, R&D Systems, dilution 1/500) and GAPDH (0411, Santa Cruz Biotechnology, dilution 1/5000). Then, membranes were washed and incubated for 1 h at RT with the corresponding peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Specific signals were detected using the enhanced chemiluminescence (ECL) detection method.

2.7 Cell viability assay

The biocompatibility effect of free FRT or loaded in NPs on MCTS, was evaluated by measuring the ATP content of the cells, using the CellTiter-Glo® 3D Reagent (Promega Corporation). Aliquots of 250 cells/well were seeded in the ULA plates for cell viability assays. After 5 days, when the gastric MCTS were formed, cells were treated with B72.3 functionalized FRT-loaded PLGA-PEG-COOH NPs (NFB72.3) diluted in cell culture medium serum-free at concentrations ranging from 0,1 to 5 µM for 24 h. Following that, we compared the treatment with 1 µM of free FRT, 1 µM of IgG Functionalized FRT-loaded PLGA-PEG-COOH NPs (NFIgG) and 1 µM of NFB72.3 during 2 timepoints: 24h and 36h. Finally, to evaluate the effect of PLGA-PEG NPs and B72.3 antibody on biocompatibility on MCTS, we incubate them during 72h. After the timepoint finished, 50 µL of medium was removed and 50 µL CellTiter-Glo® 3D Reagent were added. MCTS were destroyed by mechanical force during 5 min and incubated for 25min at RT protected from light. Luminescence was measured using a SynergyMx™ MultiMode Microplate Reader (BioTek™, USA). All samples were performed at least in triplicates.

2.8 In vivo treatments

All animal experiments were performed in strict accordance with the recommendations of the European Union Directive 2010/63/EU, following a protocol previously approved by the i3S Animal Ethics Committee and the Competent Authority in Portugal (Direção-Geral de Alimentação e Veterinária (DGAV)) (reference 2021_05) concerning the humane endpoints, appropriate husbandry and protection of experimental animals.

Female and male N:NIH(s)II:nu/nu nude mice, aged 9-10 weeks and weighing 16–20 g and 20-26 g, respectively, were bred and cared at the i3S Animal Facility (Porto,

Portugal) being fed by *ad libidum* and supplemented with Gel Boost (H₂O Clear) and Anima Strath. To generate tumor xenografts, animals were subcutaneously injected in the scapula area, with 7.5×10^5 MKN45 SC cells, resuspended in 100 μ L serum-free RPMI. When the tumor volumes reached between 50-70mm³, MKN45 SC xenograft mice were randomized into 4 groups ($n = 7-10$ per group) and treated with 100 μ L of (a) saline/PBS (CTR); (b) free FRT; (c) NFIgG; (d) NFB72.3; total dose: 30 mg/kg mice.

Animals were intravenously injected in the lateral tail vein, every two days for 2 weeks (total of 6 times). Tumor volume was measured every two days during the treatment period, using a caliper and calculated as $V = \frac{1}{2} (\text{length} \times \text{width}^2) (\text{mm}^3)$ [47]. Relative tumor growth (RTG) was calculated according to the following formula: $\text{RTG} = \text{TV}_n / \text{TV}_0$ where TV_n is the tumor volume at the day of measurement and TV_0 is the tumor volume on the first day of measurement [48]. Mice were weighted twice a week during the period of the study.

At the end of the study, the mice were euthanized by cervical dislocation after isoflurane anesthesia and the tumors were collected and divided: half of the tumor was frozen for immunofluorescence analysis and the other half was fixed for 48h in formalin and afterwards embedded in paraffin, sectioned into 5 μ m thickness sections, and stained with hematoxylin and eosin. The humane endpoints that we considerate under the study were a maximum of 20% of weight loss or a tumor size of 2000 mm³.

2.9 Immunohistochemical analysis of tumor xenograft

Expression of STn (B72.3; undiluted), Citokeratin (AE1/AE3, Menarini Diagnostics; dilution 1:5000) and Ki-67 (D2H10, Cell Signaling Technology; dilution 1:500) were assessed in paraffined tumor mice xenograft tissues (4 μ m). Heat mediated antigen retrieval using sodium citrate buffer (10 mM, pH 6.0) was required for the Citokeratin and Ki-67 markers. Endogenous peroxidase blocking was performed in all the samples using 3% hydrogen peroxide. Tissues were incubated overnight with the respective primary antibodies diluted in 5% BSA, PBS, followed by biotin-labeled secondary antibodies (Dako) and with ABC kit (Vector Laboratories). Sections were stained with DAB (Sigma-Aldrich) containing 0.01% H₂O₂ and counterstained with Mayers' hematoxylin. Images were taken under 40 $\times$ magnification with a Zeiss Optical Microscope.

2.10 Immunofluorescence of tumor xenograft

Expression of p-MET (Y1234/1235, Cell Signaling Technology; dilution 1:100) and p-Ron (Y1238/Y1239, R&D Systems; dilution 1:100) were assessed in frozen tumor mice xenograft tissues (10 μ m). The tissues were fixed with 4% PFA for 15 min at RT. Fixed samples were permeabilized with Triton 0,5% Triton X-100-PBS for 10 min at 4°C. In brief, slides were blocked with Animal-Free Blocker (Vector Laboratories) and incubated overnight with the corresponding dilution of the primary rabbit antibodies. Slides were then washed with PBS and incubated with the secondary antibody anti-rabbit IgG conjugated with Alexa Fluor®-594 (dilution 1:500) for 30 min at RT. Nuclei were counterstained with DAPI (dilution 1:100) and samples were mounted with VectaShield (Vector Laboratories). Images were acquired at 20x magnification with Zeiss Axio Imager Z1 microscope equipped with an AxioCam MR ver.3.0 (Carl Zeiss).

2.11 Imaging analysis

For tissues of tumor mice xenografts, images were taken at 40x magnification using a Zeiss Optical Microscope, having at least 4 images of each tumor tissue and analyzed using ImageJ software with the Fiji image-processing package [49]. The intratumoral proliferative index was determined by calculating the fraction Ki-67 positive cells. First, all images were preprocessed with a median filter to reduce noise, followed by color deconvolution (using “H-DAB” color vectors) to separate, into distinct channels, the brown positive cells (Ki-67) from the remaining blue positive cells. Image segmentation was performed on each channel using Otsu’s, threshold method, and the watershed operation was used to resolve adjacent cells. The mask for the blue positive cells was further improved using the close and fill holes morphological operators. Morphometric analysis was then applied (using Fiji’s “Analyze Particles” tool), and all objects smaller than 1000 pixels and with circularity outside the range 0.5-1.0 were removed. The resulting masks were then used to define and quantify the blue positive cells and the brown positive cells (Ki-67) by ImageJ’s shape descriptor. The image analysis workflow was validated using measurements from a manually annotated images dataset (“gold-standard”) obtained from two independent users.

The intratumoral necrosis area was determined in slides stained with hematoxylin and eosin and delimiting manually the area of necrosis versus the area of tumor and calculating with the software.

The fluorescent staining from pMET and pRON activation upon NPs treatment of the tumor mice xenografts were analyzed by applying the “color split channels” function to

subdivide the red and blue channels. The pixels of each group were quantified applying the “histogram” function and obtaining the mean of intensity. At least 3 different images per condition were analyzed. We normalized the signal:

$$(\%) \text{ phosphorylation} = (\text{RED mean intensity} / \text{BLUE mean intensity}) * 100$$

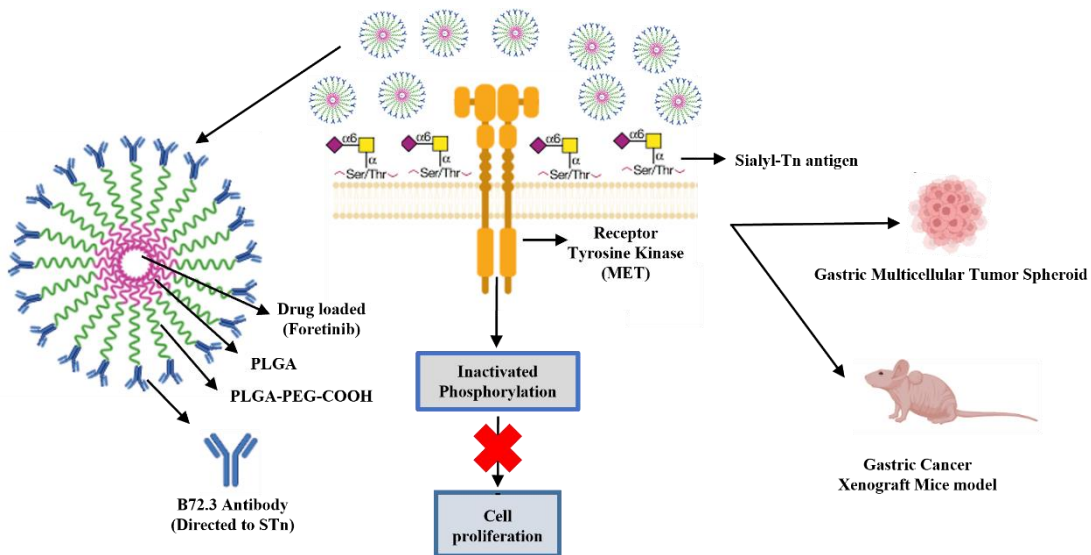
2.12 Statistical analysis

Results are expressed as mean $\pm$ standard deviations (SD) of three independent experiments. Data were analyzed using GraphPad Prism 8.1 software (GraphPad, San Diego, CA, USA). Statistical analysis was performed by ANOVA for multiple group comparison and Student’s t-test for the individual group comparison. The level of significance was set at probabilities of *p < 0.05; **p < 0.01; ***p < 0.001 and ****p<0.0001.

3 Results

3.1 Development and characterization of an antibody-conjugated NP loaded with Foretinib

To develop antibody-conjugated NPs, we initially produced the PLGA-PEG-COOH NPs loaded with FRT (NF). Then we proceeded with the functionalization of the NPs with the antibody B72.3 targeting STn (**Scheme 1**), using carbodiimide chemistry method. A NP functionalized with an unspecific IgG1 antibody was used as a non-targeted control NP.



Scheme 1: Schematic representation of Foretinib loaded NPs functionalized with B72.3 anti-Sialyl-Tn mAb. These NPs directed to Sialyl-Tn, act at the cell membrane, close to the receptor tyrosine kinase, for an efficient drug uptake.

Detailed characterization of the developed NPs included physicochemical characteristics are summarized in **Table 1**. All the prepared polymeric NPs had low polydispersity index (Pdl) values, ranging between 0.08-0.21. The size of our formulations ranged between 126 and 199 nm. All formulations were characterized as negatively charged, between -17 and -6.9. The conjugation efficacy (CE), quantified by LC-MS, of the functionalized FRT-loaded NPs showed 92.3% in B72.3 functionalized FRT-loaded PLGA-PEG-COOH NPs (NFB72.3) and 95.3% IgG functionalized FRT-loaded PLGA-PEG-COOH NPs (NFIgG) (**Table 1**). The results of drug association efficiency (AE) and drug loading (DL) are also presented in **Table 1**. We demonstrated a successful encapsulation of FRT with 90.2% of AE and 4.29% of DL, for non-functionalized NF. For functionalized NF, NFB72.3 presented AE and DL of 89.4 and 4.26, and NFIgG presented 84.1 and 4.0, respectively. The surface morphology and size distribution of NPs were evaluated by transmission electron microscopy (TEM) (**Fig. S1**, Supplementary material), revealing that NPs are spherical and with uniform size distribution, in agreement with the DLS results.

Table 1: Properties of functionalized and non-functionalized nanoparticles, including the mean size, polydispersity index (Pdl), surface charge, association efficacy (AE), drug loading (DL) and conjugation efficacy (CE). The values were represented as mean values $\pm$ SD (N=3).

	Size (nm)	Pdl	Surface Charge (mV)	AE (%)	DL (%)	CE (%) ELISA	CE (%) LC-MS
PLGA NP (NP)	126.8 $\pm$ 4.6	0.151 $\pm$ 0.014	-6.9 $\pm$ 0.8	n.a.	n.a.	n.a.	n.a.
PLGA-PEG-COOH NP (NPP)	132.8 $\pm$ 3.0	0.181 $\pm$ 0.013	-10.8 $\pm$ 1.2	n.a.	n.a.	n.a.	n.a.
B72.3 Functionalized PLGA-PEG-COOH NP (NB72.3)	153.8 $\pm$ 2.8	0.161 $\pm$ 0.010	-15.5 $\pm$ 2.8	n.a.	n.a.	87.3 $\pm$ 1.3	n.a.
IgG Functionalized PLGA-PEG-COOH NP (NIG)	174.6 $\pm$ 1.5	0.174 $\pm$ 0.024	-15.9 $\pm$ 1.0	n.a.	n.a.	89.5 $\pm$ 2.1	n.a.
FRT-loaded PLGA-PEG-COOH NP (NF)	147.4 $\pm$ 2.7	0.088 $\pm$ 0.015	-10.9 $\pm$ 1.2	90.2 $\pm$ 1.9	4.29 $\pm$ 0.09	n.a.	n.a.
B72.3 Functionalized FRT-loaded PLGA-PEG-COOH NP (NFB72.3)	178.1 $\pm$ 4.0	0.113 $\pm$ 0.008	-16.5 $\pm$ 0.5	89.4 $\pm$ 5.4	4.26 $\pm$ 0.25	88.5 $\pm$ 3.4	92.3 $\pm$ 7.0
IgG Functionalized FRT-loaded PLGA-PEG-COOH NP (NFIgG)	199.9 $\pm$ 6.8	0.212 $\pm$ 0.053	-17.2 $\pm$ 1.7	84.1 $\pm$ 3.2	4.00 $\pm$ 0.15	91.0 $\pm$ 4.1	95.3 $\pm$ 3.7

3.2 Evaluation of the interactions between gastric cancer cells and nanoparticles

We previously demonstrated by immunoTEM, flow cytometry and immunofluorescence the targeting ability of NPs functionalized with B72.3 against gastric cancer cells that express or not STn, when compared with NP functionalized with IgG [36]. In this study, to assess the specificity and targeting ability of our nanosystem, we evaluated by flow cytometry the interaction between NFB72.3 or NFIgG and gastric MCTS from MKN45 SC and MKN45 WT cells that express or not STn, respectively (**Fig. 1A**). Both functionalized NPs (100µg/mL) were incubated for 2h with gastric MCTS. NFB72.3 showed an efficient and specific interaction capacity towards MKN45 SC, that express STn glycan (**Fig. 1A**). In addition, NFIgG did not interact with none of the cell models (**Fig. 1A**).

Then, we incubated gastric MCTS with PLGA-PEG-COOH NPs (NPP) in the absence of FRT drug during 72h and, by cell viability assays, we confirmed the lack of cellular cytotoxicity. The incubation with B72.3 antibody also did not show any cytotoxicity for gastric MCTS (**Fig. S2**, Supplementary material).

Finally, by flow cytometry we demonstrated that our gastric MCTS models had stable and similar levels of expression of STn glycan for each cell line at day 6 or at day 8 (**Fig. S3**, Supplementary material).

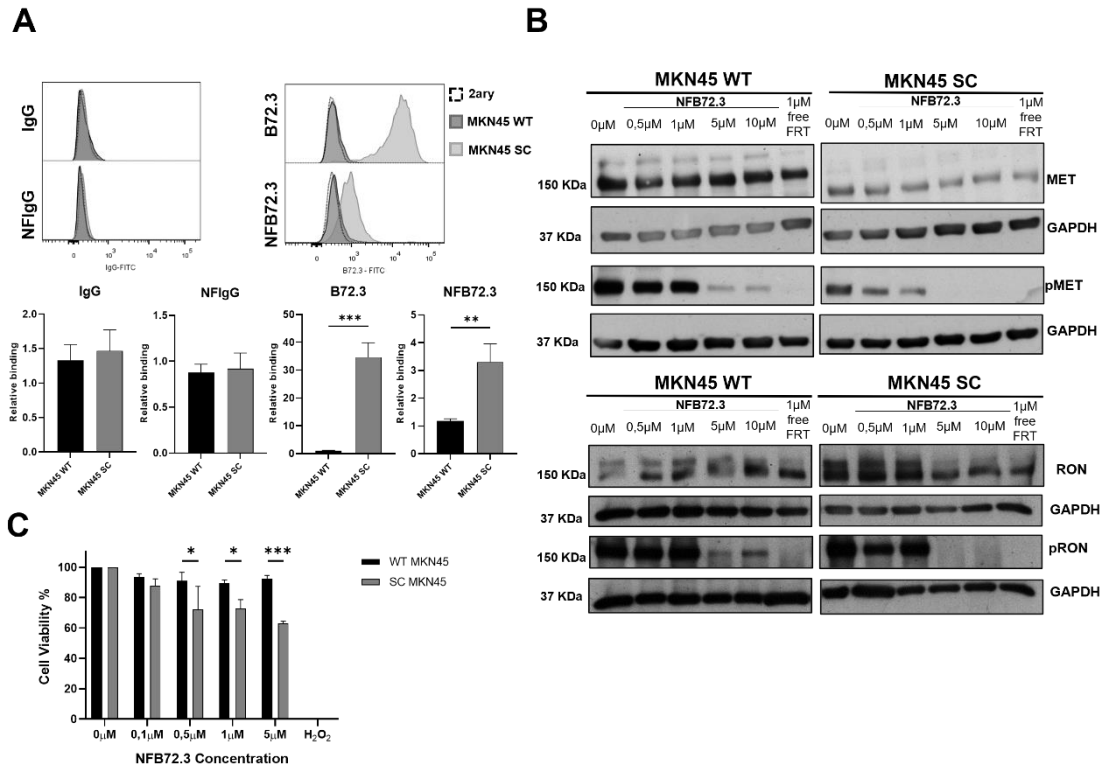


Figure 1: A) Flow cytometry analysis of MKN45 WT and MKN45 SC incubated with IgG functionalized PLGA-PEG NP (NFIgG) and B72.3 functionalized PLGA-PEG NP (NFB72.3). Data presented as representative histograms of 3 independent experiments and mean of relative binding $\pm$ SD; **B)** Western blot analysis of MET and RON receptor tyrosine kinases and their activated forms, pMET and pRON after treatment with a range of concentrations of NFB72.3 and 1 μ M of free Foretinib in MKN45 WT and MKN45 SC gastric multicellular tumor spheroids (MCTS) for 24h. GAPDH was used as a loading control; **C)** Celltiter Glo 3D cell viability assay after incubation of a range of B72.3 functionalized FRT-loaded PLGA-PEG-COOH NPs (NFB72.3) in MKN45 WT and MKN45 SC gastric MCTS during 24h.

3.3 Effect of Foretinib delivery by functionalized nanoparticles against STn in gastric cancer cells

We evaluated the effect of different concentrations of free FRT (0.01, 0.05, 0.1, 0.5, 1 and 5 μ g/mL) on MET and RON activation in MKN45 WT and MKN45 SC gastric MCTS. We observed that FRT decrease the MET activation with concentrations equal or higher than 0.1 μ g/mL (**Fig. S4**, Supplementary material). Taking this into consideration, we evaluated the effect of NFB72.3 applying a range of concentrations between 0.5-10 μ g/mL of encapsulated FRT. Our results showed that upon incubation with 1 μ g/mL of FRT encapsulated NPs there was an enhanced inactivation of both RTKs (MET and RON) in our cell models (**Fig. 1B**). In addition, we evaluated the cell viability using the same range of drug concentrations of NFB72.3, in our gastric MCTS models. None of the concentrations influenced MKN45 WT metabolic activity. However, decreased cell viability was observed in MKN45 SC when treated with 0.5; 1 and 5 μ g/mL of NFB72.3 (**Fig. 1C**). Taking into consideration these results 1 μ g/mL was selected to perform the following *in vitro* experiments.

3.4 In vitro evaluation of the specificity of functionalized NPs and drug delivery

Considering the last results that demonstrated that 1 μ g/mL of drug encapsulated in NFB72.3 was the best concentration to specifically deliver the FRT, we evaluated and compared the FRT delivery by two different functionalized NPs: NFIgG and NFB72.3. We incubated gastric MKN45 WT and MKN45 SC MCTS with 1 μ g/mL FRT in NFIgG or NFB72.3. Free FRT at the same concentration was used as positive control. We observed a very expressive inactivation of MET and RON phosphorylation in MKN45 SC MCTS upon treatment with NFB72.3 functional nanosystem at 24h. This was not observed in MKN45 WT MCTS. In contrast, no differences in MET and RON activation

were observed in shorter (6h) and longer (36h) times of incubation (**Fig. S5**, Supplementary material). These results demonstrate the efficient targeting and selective ability of these NPs to deliver the drug after 24h of treatment.

Following this, we performed an *in vitro* cell viability evaluation in gastric MCTS through CellTiter-Glo upon incubation with 1 µg/mL of free FRT, NFIgG or NFB72.3 for 24h and 36h. The obtained results showed a decrease in cell viability of both gastric MCTS when treated with NFB72.3 that was more significant at 36h incubation (**Fig. 2C**).

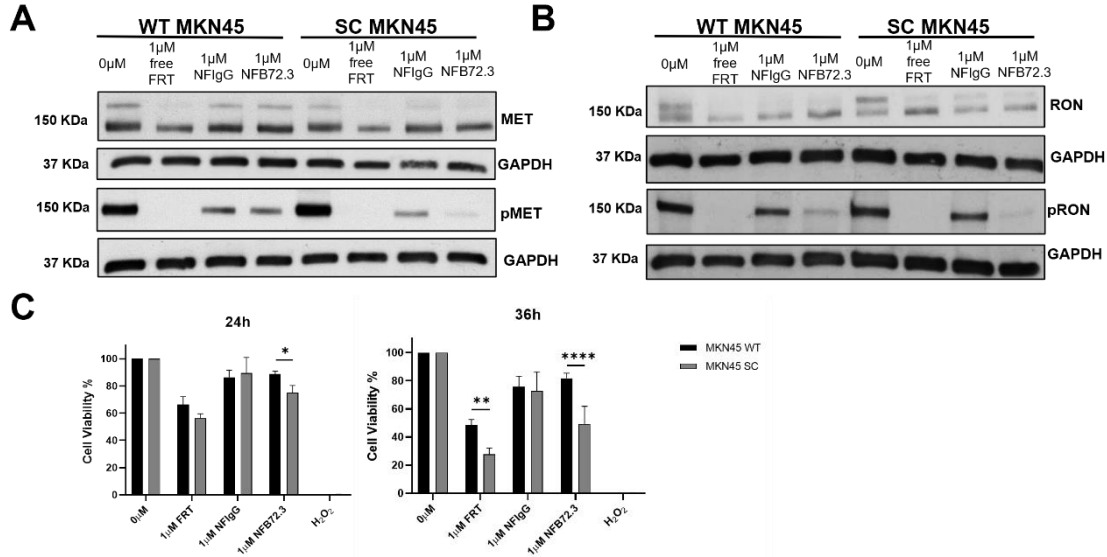


Figure 2: Western blot analysis of **(A)** MET and **(B)** RON receptor tyrosine kinases and the activated form, **(A)** pMET and **(B)** pRON, after treatment with 1µM of concentration of free or encapsulated Foretinib in functionalized nanoparticles (NFIgG or NFB72.3) in MKN45 WT and MKN45 SC gastric multicellular tumor spheroids (MCTS) during 24h. GAPDH was used as a loading control. **(C)** Celltiter Glo 3D cell viability assay after incubation of 1µM of free Foretinib, IgG Functionalized FRT-loaded PLGA-PEG-COOH NPs (NFIgG) and B72.3 Functionalized FRT-loaded PLGA-PEG-COOH NPs (NFB72.3) in SC MKN45 and WT MKN45 gastric multicellular tumor spheroids (MCTS) during 24h and 36h.

3.5 Evaluation of Foretinib delivery by functionalized nanoparticles in tumor growth

Taken into consideration the *in vitro* results, we then determined the *in vivo* antitumor efficacy of our bioconjugate NFB72.3 in nude mice subcutaneously injected with MKN45 SC cells. After tumors had reached a size of 50-70 mm³, we performed comparative efficacy studies. Animals were divided randomly in four groups ($n = 7 - 10$ per group), in a way to minimize weight and tumor size differences among the groups. Animals were intravenously treated with saline/PBS (CTR), free FRT, NFIgG or NFB72.3 every two days for 2 weeks (total of 6 times of injection), using the previously reported dose of

30mg/kg for FRT [10, 12-14]. After 48h of the last treatment, tumors were collected, sized and further divided to be frozen or paraffin-embedded (**Fig. 3A** and **B**). Tumor volume, relative tumor growth (RTG) and response to treatment measurements are shown in **Fig. 3C** and **E**. The treatment did not show any efficacy for the mice treated with PBS, free FRT or NFIgG, and at the end of the study the mean size for these groups were respectively $543.86 \pm 87.49 \text{ mm}^3$, $512.24 \pm 131.73 \text{ mm}^3$, $557.41 \pm 127.44 \text{ mm}^3$ (**Fig. 3B** and **C**). None of the animals from these groups exhibited tumor regression. In contrast, NFB72.3-treated group, showed a decrease in tumor growth, with a final mean tumor size of $354.53 \pm 47.63 \text{ mm}^3$ (**Fig. 3B** and **C**). The NFB72.3-treated group demonstrated significant reduction in tumor volume and relative tumor growth when compared to those treated with free FRT or NFIgG, 13 days after the first treatment (**Fig. 3D** and **F**). Overall, NFB72.3 exhibited a therapeutic effect by slowing MKN45 SC primary tumor growth.

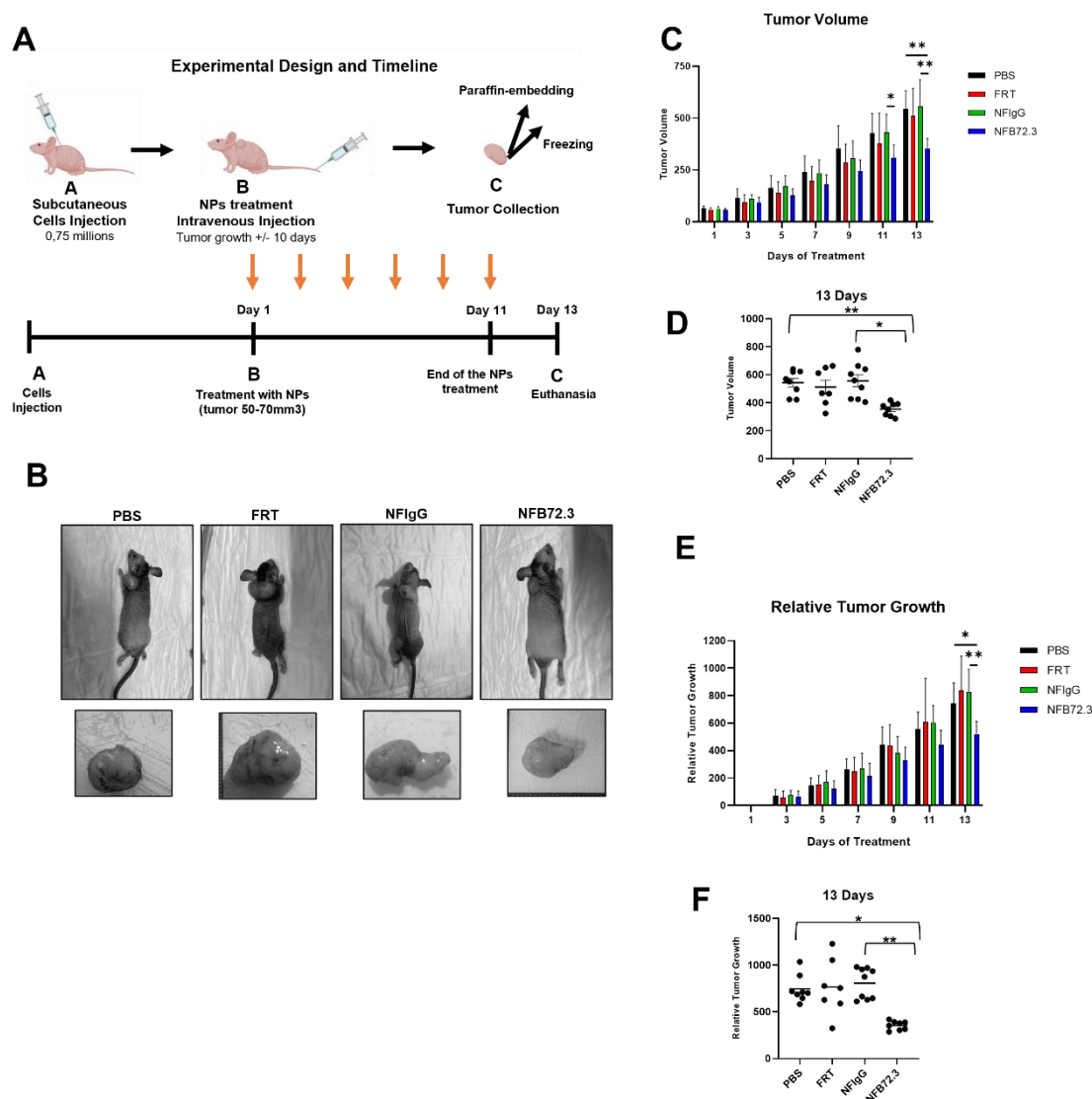


Figure 3: **A)** Experimental design of the *in vivo* experiment. **B)** Mice images were taken at day of euthanasia as well as the respective mice tumor xenografts images, after being removed by surgery, 13 days after first treatment administration. **C)** Tumor volume (mm³) measurements were performed using a caliper before any treatment, during the days of treatment until animals being euthanized. **D)** Tumor volume (mm³) measurements at the day of euthanasia. **E)** Relative tumor growth was calculated by dividing the tumor volume at measurement day by the tumor volume before starting the treatment. **F)** Relative tumor growth measurements of the animals in the day of euthanasia.

3.6 Histopathologic characterization of xenograft tumors treated with functionalized nanoparticles loaded with Foretinib

MKN45 SC Xenograft tumors treated with NFB72.3, NFIgG, free FRT and PBS control were characterized by immunohistochemistry, confirming the presence of epithelial tumor cells stained by cytokeratin marker. In all these tumors we also validate the expression of STn antigen (**Fig. 4A**). The tumors were also analyzed by immunohistochemistry for the Ki-67 proliferative marker, and it was observed a significantly lower number of carcinoma cells expressing Ki-67 (% proliferating cell) in the NFB72.3 treated group compared to the control group (PBS). MKN45 SC Xenograft tumors treated with FRT alone or NFIgG did not show any reduction in the proliferative index compared to the control (PBS). These results indicated that NFB72.3 treatment had stronger *in vivo* antiproliferative effect compared to free FRT or FRT loaded in a NP functionalized with an antibody that did not target these cells. (**Fig. 4A and B**)

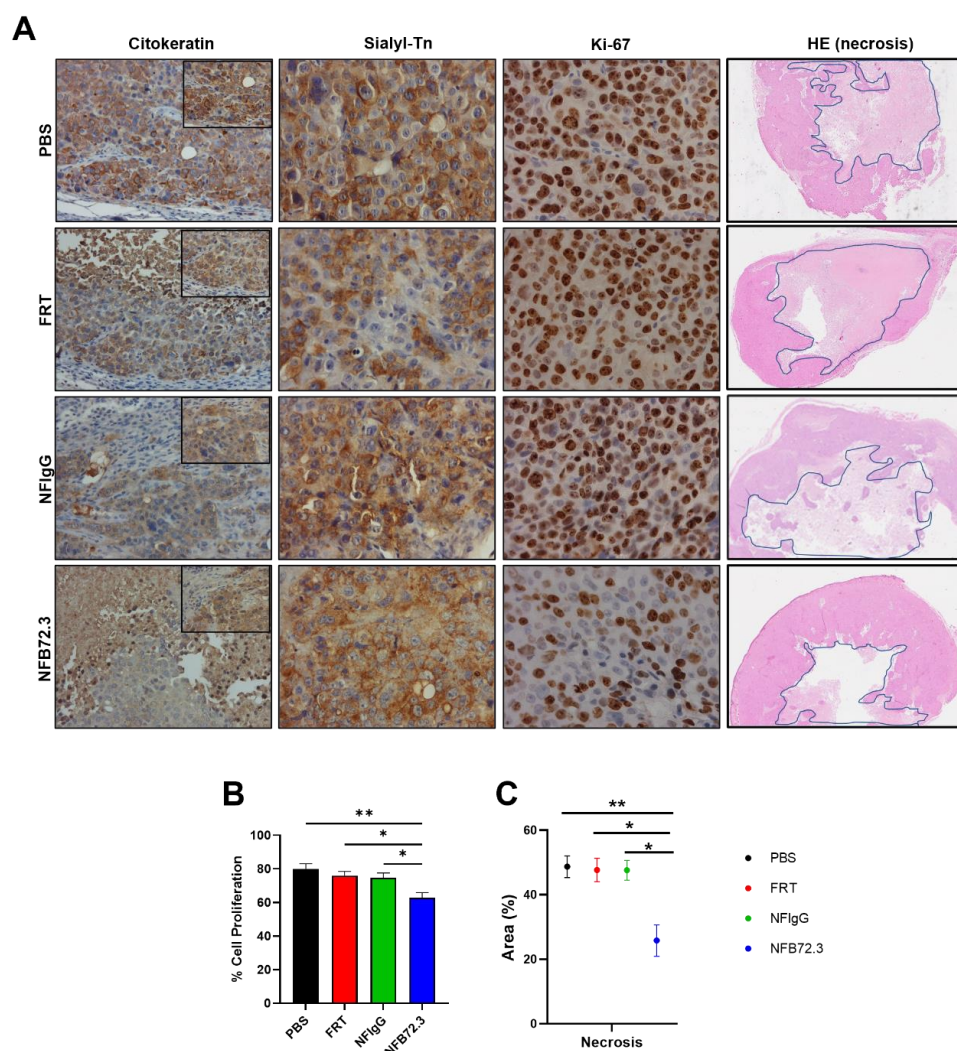


Figure 4: **A)** Xenografts from MKN45 SC-injected mice clearly presented expression of Cytokeratin and Sialyl-Tn. Different patterns of Ki-67 staining were observed according with the treatment and the representative area of necrosis was highlighter in the HEs. Images were taken under 20x magnification for for Cytokeratin and hematoxylin and eosin (HE) and at 40x magnification for Sialyl-Tn, Ki-67 and Cytokeratin insert. **B)** Intratumoral proliferation was immunohistochemical measured by Ki-67 nuclear antigen in the tissue sections. Ki67-positive cells were counted in ImageJ software. **C)** Area of necrosis was measured in HE tissues sections and was calculated with ImageJ software.

Then, we quantified the area of tumor cells and necrosis (**Fig. 4A**) in hematoxylin and eosin (HE) stained tissues. We observed that tumors treated with NFB72.3 presented significantly smaller percentage of area of necrosis (25.80 ± 11.93), comparing to the other groups (PBS 48.72 ± 8.27 , FRT 47.70 ± 6.32 , NFIgG 47.62 ± 6.89) (**Fig. 4C**). These results suggest that the tumor xenografts treated with NFB72.3 NPs did not grow so fast as in the other groups, which is in agreement with the results obtained for tumor growth (**Fig 3B**).

3.7 Effect of functionalized nanoparticles loaded with Foretinib on MET/RON phosphorylation *in vivo*

The effect of NFB72.3 treatment on MET and RON phosphorylation was assessed *in vivo* using the MKN45 SC xenograft mouse model. A significant decrease in the percentage of activation of phospho MET and phospho RON was observed in the NFB72.3 treated group, 6.32 ± 3.23 and 7.85 ± 3.74 , respectively (**Fig. 5A-B**). The mean of percentage of activation of phospho MET and phospho RON for each group control was 65.03 ± 19.18 and 70.71 ± 9.48 , respectively, for PBS, 31.53 ± 6.43 and 58.24 ± 16.53 , respectively, for FRT and 63.96 ± 13.85 and 75.39 ± 29.25 , respectively, for NFIgG (**Fig. 5B**). These results demonstrated that NFB72.3 treatment in MKN45 SC xenografts tumor tissues was efficient to inhibit the activation of phospho MET and phospho RON.

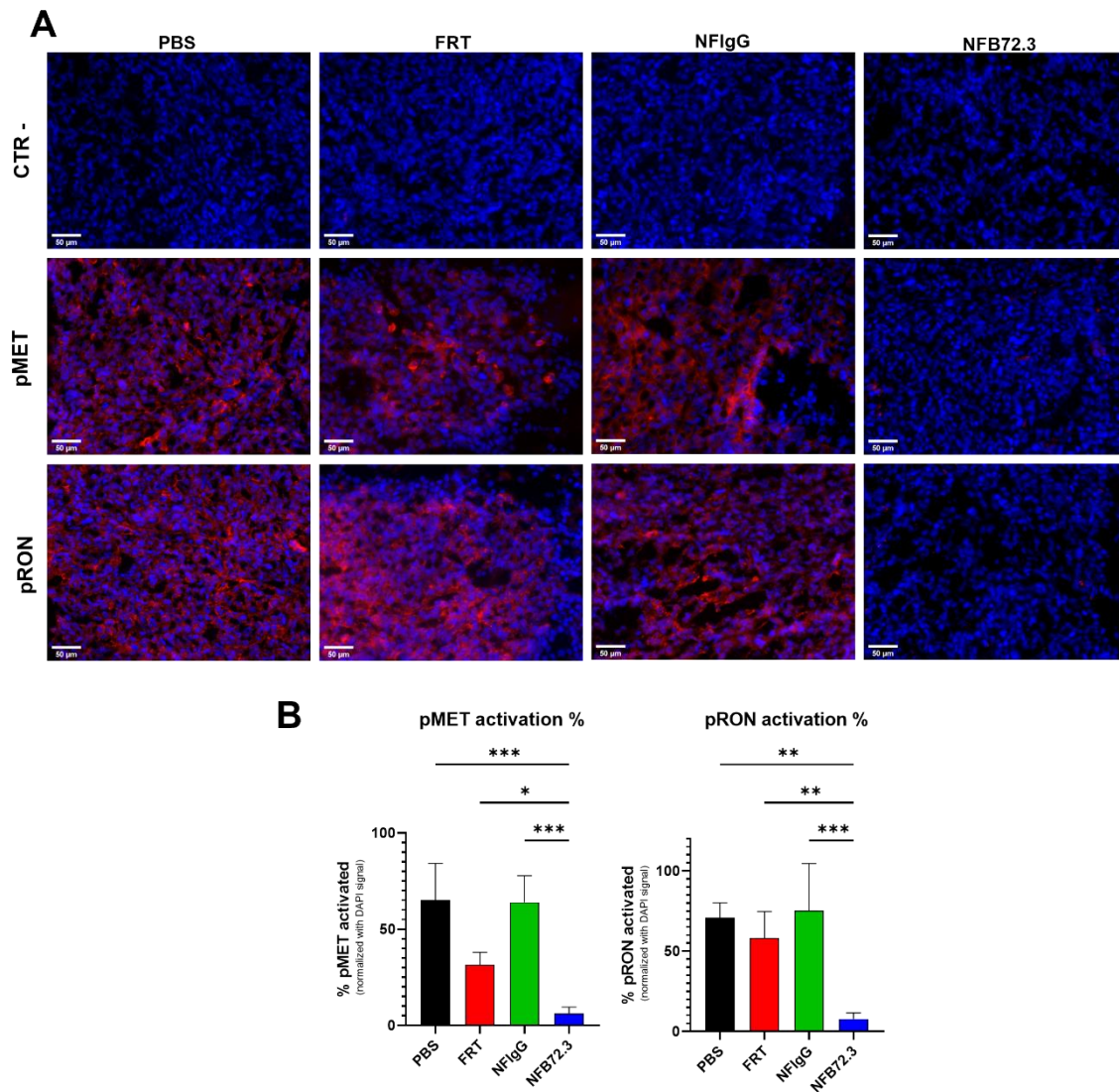


Figure 5: *In vivo* effect of FRT, NFIgG and NFB72.3 treatment on the activation of **(A)** phospho MET and phospho RON by immunofluorescence; **(B)** quantification of activation of phospho MET and phospho RON. Images were taken under 20x magnification.

4 Discussion

Effective treatment of GC remains a major therapeutic challenge [50]. Targeted therapies using TKI are an emerging promising approach in the clinical setting [51]. Recently, efforts to improve drug delivery with less toxicity has led to the development of functional nanosystems as they increase the potential for an effective delivery of the drug in cancer therapy [23, 25]. In this study, we developed a nanodelivery system that takes advantage of the aberrant sialylated glycans present in GC cells to specifically deliver TKI to target cells, with potential to be used for GC treatment. The novel NPs were developed and loaded with FRT using pegylated PLGA NPs functionalized with an antibody targeting the STn antigen. This cancer-associated carbohydrate antigen is highly expressed in cancer cells and essentially absent in normal cells [31]. The potential of this novel NPs was further validated using *in vitro* and *in vivo* models (**Scheme 1**).

The conjugation of B72.3 antibody to the PLGA-PEG-COOH block copolymer have been successfully established by our group. These bioconjugates were shown to be highly specific to STn expressing cancer cells [36]. In agreement with this, in our present study we produced these NPs applying the same functionalization strategy and obtained similar CE values as determined by ELISA and LC-MS (**Table 1**). These results demonstrate the efficacy of the strategy using the covalent link between the amine group of the antibody and the carboxylic group from the PEG copolymer. Moreover, the hydrophilic PEG block promotes the presentation of the carboxylic acid on the NP surface, increasing the number of immunoglobulins that were covalently linked [52, 53]. The production of polymeric nanoparticles by nanoprecipitation has been described as the best strategy to encapsulate hydrophobic drugs, such as FRT [54].

In general, the physicochemical characterization and morphology (**Table 1** and **Fig. S1**) of NFB72.3 revealed, as expected, that they are suitable for an effective use. The size of our NP formulations, ranged between 126 and 199 nm, has been described as ideal for intravenous administration. This size of NPs have been shown to be able to infiltrate through the tumor vasculature, avoiding the normal vasculature [55]. All formulations presented PdI values lower than 0.21, indicating a relative homogenous size distribution that is frequently observed for polymeric NPs (**Table 1**). Regarding the values of AE and

DL of 1mg of FRT in NF, NFB72.3 and NFIgG, they were obtained by LC-MS quantification and revealed to be very satisfactory, ranging between 84.1 – 90.2 % of AE and 4.00 - 4.29 % of DL (**Table 1**). All these results pointed towards the appropriated range of values and, therefore, administration of higher doses was avoided to consequently avoid drug side effects. Previous studies using polymeric NPs loaded with FRT exhibited a PDI of 0.21 and an AE of 37% of 1 mg [56], demonstrating that our FRT loading system was highly efficient. Considering the shape of the NPs (**Fig. S1**), our formulations presented a spherical and uniform size distribution, which has been associated with faster cell internalization [57].

Our *in vitro* studies for the evaluation of the cell-NP interaction validated the specific targeting ability of this nanosystem. MCTS have been previously shown to be a good model to better mimic the tumor microenvironment [45, 46] (**Fig. 1A**). Our data showed that NPs functionalized with the anti-STn antibody specifically targeted MCTS of GC cells expressing STn (MKN45 SC). Moreover, NPs functionalized with IgG showed no interaction with GC cells (**Fig. 1A**).

In addition, our evaluation of the delivery effect of FRT showed that 1 µg/mL of drug loaded in the NFB72.3 was efficient to inactivate the RTKs MET and RON upon 24h of incubation (**Fig. 1B**). Previous studies have shown that FRT has MET receptor as a preferential target [9, 13, 15]. Our results are in agreement, as the highest effect exerted by the NPs loaded with FRT was observed in this TKR (**Fig. 1**).

Moreover, *in vitro* cell viability assays were performed during 24h to confirm the impact of NFB72.3 in MKN45 SC and MKN45 WT cells. As expected, NPs revealed to specifically target STn-positive cells as no effect on cell viability was observed for the MKN45 WT (**Fig. 1C**). Interestingly, a clear decreased in cell viability was observed in MKN45 SC when treated with NFB72.3 (**Fig. 1C**). NFIgG NPs, that do not target STn antigen, did not show any effect in the activation of phospho MET and phospho RON in both GC cell models (**Fig. 2A and B**). These results support the specific targeting ability of our NPs, demonstrating their capacity for being uptake and promote the delivery of the drug to the target cell. Further MCTS cell viability assays using NFB72.3 and NFIgG NPs demonstrated that NFB72.3 specifically affect the cell viability of MKN45 SC contrarily to MKN45 WT (**Fig. 2C**). In contrast, NFIgG displayed no significant effect in the viability of both cell models (**Fig. 2C**). Overall, these findings strongly support the safety and specificity of this novel nanosystem functionalized against STn and highlight its therapeutic potential *in vivo*.

The analysis of the therapeutic effect *in vivo* demonstrated that the intravenous administration of NFB72.3 induced a decrease in MKN45 SC tumor growth compared to the treatment with free FRT or NFIgG (**Fig. 3C** and **E**). These results support the importance of loading FRT in NPs functionalized with an antibody targeting STn positive cancer cells, when compared with its free delivery or in NPs lacking an STn antibody. To the best of our knowledge, this is the first study demonstrating the specific delivery of FRT encapsulated in NPs functionalized with an antibody directed to STn and validated in cancer mouse models. However, previous studies with free FRT have been performed *in vivo* in pancreatic [12], esophageal [11] and endometrial [13] cancer. Of note, our study showed no effect on tumor growth reduction in the group treated with free FRT when compared to NFB72.3, contrary to what was observed in previous studies [11-13]. These differences are probably due to the fact that we used a different via of drug administration and different treatment scheme, including less days of treatment. Importantly, we demonstrated that when we encapsulate FRT in polymeric NPs that were functionalized to target STn, we end up with a nanodelivery system able to decelerate the tumor growth (**Fig. 3C**). Further validation of our nanosystem by intravenous injection will demonstrate its applicability in a clinical setting.

Detailed analysis of our *in vivo* study showed that treatment with NFB72.3 consistently decreased tumor cell proliferation and necrosis area (**Fig. 4B** and **C**). This was not observed for the other treated groups, including free FRT, which is in agreement with Hassan *et al.* that showed that esophageal adenocarcinoma xenograft tumors treated with free FRT did not decrease tumor cell proliferation [10]. Our results further support the potential usage of NP directed to STn expressing cancer cells as an effective delivery system of FRT.

In addition, we assessed the effect of our innovative therapy approach in MET and RON phosphorylation in the tumor tissues. Our data demonstrated that NFB72.3 efficiently inhibited the MET and RON phosphorylation, contrarily to the other groups (**Fig. 5A** and **B**). Similarly, other reports demonstrated the same results when combined FRT with one more therapeutic drug [10, 58]. In addition, Kogata *et al.* reported in endometrial cancer xenografts inactivation of phospho MET treated with 30 mg/kg [13]. Although they used the same NP concentration, the scheme of administration was different, since it was administrated orally daily during 14 days [13]. Therefore, our data showed that functionalizing FRT loaded NPs with antibodies against STn represents a new potential targeted therapy regimen in MET activated gastric cancer.

5 Conclusions

In this study, we have successfully developed an innovative nanosystem directed to cancer cells expressing STn. These NPs were loaded with the tyrosine kinase inhibitor Foretinib and showed to efficiently and specifically deliver the drug to cancer spheroids of gastric carcinoma cells expressing STn. Interestingly, these NPs demonstrated a significantly decrease of the tumor growth of gastric cancer mouse xenografts expressing STn.

These results highlight that the developed NFB72.3 nanoparticle targeting the cancer-associated STn glycan structure hold a relevant potential to efficiently delivery drugs to STn-expressing gastric tumors. Finally, and since STn is expressed in tumors of several organs, this novel NP approach might also be a suitable system for other drug delivery applications in different cancers settings.

Appendix A. Supplementary Material

Figure S1: Images of empty PLGA (A), empty PLGA-PEG (B), FRT-loaded PLGA-PEG (C), B72.3 Functionalized FRT-loaded PLGA-PEG (D) and IgG Functionalized FRT-loaded PLGA-PEG (E) nanoparticles obtained by Transmission Electron Microscopy (TEM). Scale bar: 200 nm.

Figure S2: Cell viability of MKN45 WT and MKN45 SC gastric multicellular tumor spheroids (MCTS) after 72h incubation with PLGA-PEG-COOH nanoparticles and B72.3 antibody directed to Sialyl-Tn (STn), using Celltiter Glo 3D assay.

Figure S3: Sialyl-Tn (STn) level of expression (B72.3 antibody) in SC MKN45 and WT MKN45 gastric multicellular tumor spheroids (MCTS), growing for 6 days or 8 days. Data are presented as one representative flow cytometry histogram and median fluorescent intensity (MFI) normalized for binding of secondary antibody or unstained control.

Figure S4: Western blot analysis of MET receptor tyrosine kinases and activated form, phospho MET, after treatment with a range of concentrations of Foretinib for 24h, in gastric multicellular tumor spheroids (MCTS). GAPDH was used as a loading control.

Figure S5: Western blot analysis of MET and RON receptor tyrosine kinases and the activated form, phospho MET and phospho RON, after treatment with 1 μ M of concentration of free Foretinib or encapsulated in nanoparticles (NFIgG or NFB72.3) during 6h or 36h, in gastric multicellular tumor spheroids (MCTS). GAPDH was used as a loading control.

CRedit authorship contribution statement

Conceptualization, F.D., B.S., C.A.R., J.G.; Methodology, F.D., S.L., H.O., D.F., and J.G.; Histology analysis, F.G.; Software developing, P.A.; Formal analysis, all authors; Writing - original draft preparation, F.D.; Writing - review and editing, all authors; Figures and schemes, F.D.; Supervision, J.G., C.A.R., B.S.; Project administration and funding acquisition, B.S.; C.A.R.

Notes

The authors declare no competing financial interest.

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Nanoparticles targeting Sialyl-Tn for tyrosine kinase inhibitor efficient delivery in gastric cancer

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Supplemental Files (SF) for Publication

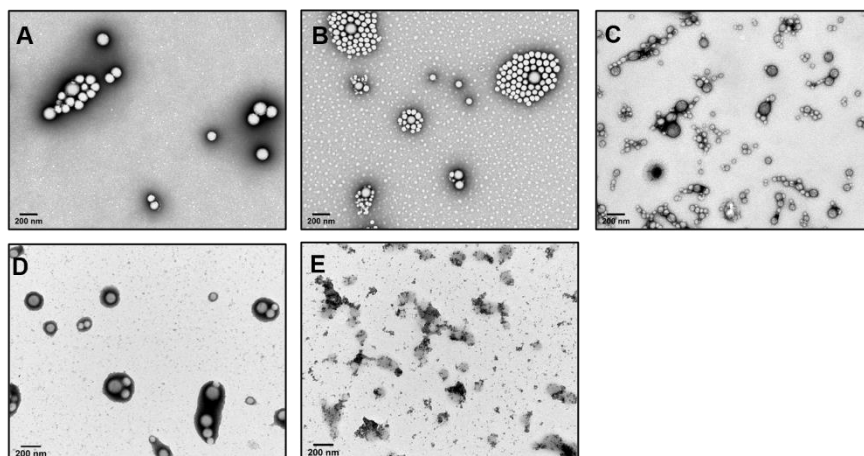


Figure S1: Images of empty PLGA (A), empty PLGA-PEG (B), FRT-loaded PLGA-PEG (C), B72.3 Functionalized FRT-loaded PLGA-PEG (D) and IgG Functionalized FRT-loaded PLGA-PEG (E) nanoparticles obtained by Transmission Electron Microscopy (TEM). Scale bar: 200 nm.

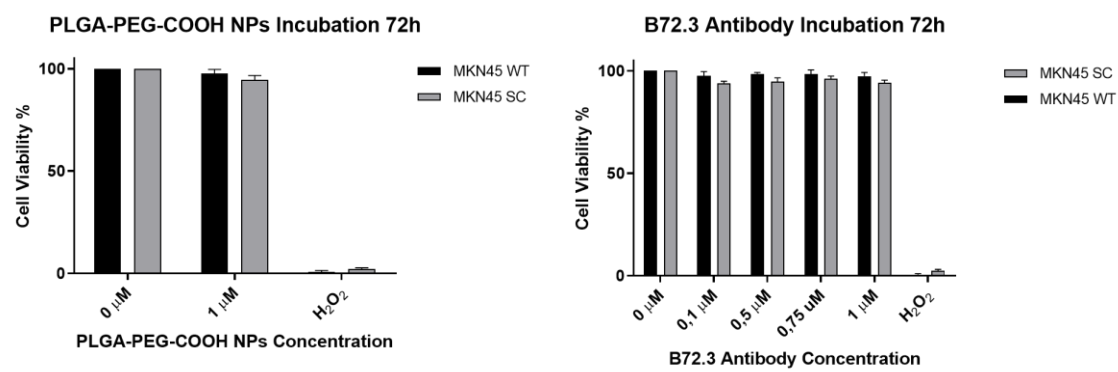


Figure S2: Cell viability of MKN45 WT and MKN45 SC gastric multicellular tumor spheroids (MCTS) after 72h incubation with PLGA-PEG-COOH nanoparticles and B72.3 antibody directed to Sialyl-Tn (STn), using Celltiter Glo 3D assay.

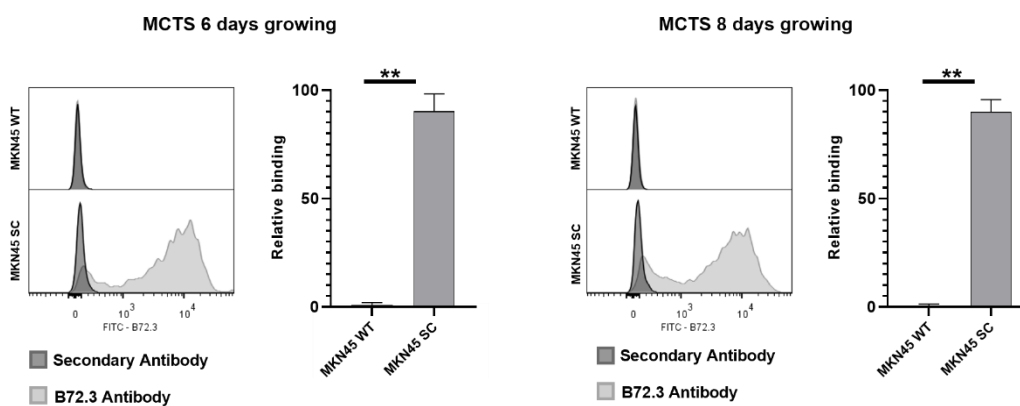


Figure S3: Sialyl-Tn (STn) level of expression (B72.3 antibody) in SC MKN45 and WT MKN45 gastric multicellular tumor spheroids (MCTS), growing for 6 days or 8 days. Data are presented as one representative flow cytometry histogram and median fluorescent intensity (MFI) normalized for binding of secondary antibody or unstained control.

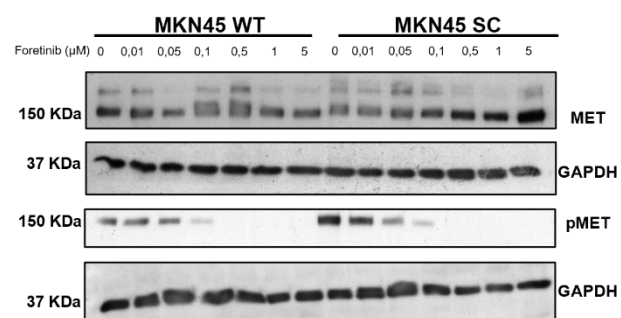


Figure S4: Western blot analysis of MET receptor tyrosine kinases and activated form, phospho MET, after treatment with a range of concentrations of Foretinib for 24h, in gastric multicellular tumor spheroids (MCTS). GAPDH was used as a loading control.

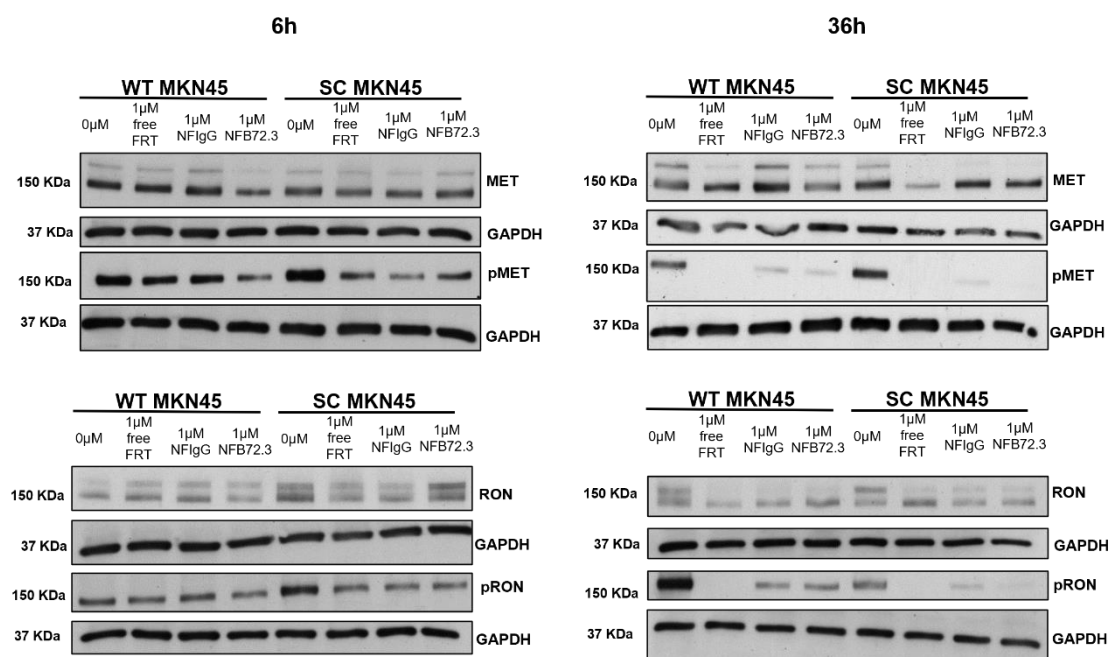


Figure S5: Western blot analysis of MET and RON receptor tyrosine kinases and the activated form, phospho MET and phospho RON, after treatment with 1μM of concentration of free Foretinib or encapsulated in nanoparticles (NFIgG or NFB72.3) during 6h or 36h, in gastric multicellular tumor spheroids (MCTS). GAPDH was used as a loading control.

CHAPTER 4

General Discussion

Conclusions

Future Perspectives

GENERAL DISCUSSION

On a global scale, is predicted that 34 million new cases will occur in 2070, this represent twice the number of cases estimated in 2018(1). Demographic and risk factor-related changes continue to have a pronounced effect on the current dimension and profile of the cancer burden worldwide, particularly among countries more developed over the past half-century. Owing to the diversity of causes across cancer types such as the long latency of the disease, and delays between effective preventative interventions and the resulting population benefit, is complex prevent properly the cancer disease. Moreover, the therapeutic offer that already exists is not the most efficient(1-4).

GC constitutes the fourth most mortal and the fifth most incident cancer disease worldwide(5). In the most recent decades, there was a decreasing trend in GC rates in many populations(1, 6). It has been proposed that this decrease could be associated with food diet, especially refrigeration, the abundance of fresh fruit and vegetables in the diet and the reduce use of salt, as well as a decrease in the use of tobacco. Nevertheless, not all types of GC are declining. Moreover, it has been reported recently a mysterious increase in GC new cases in younger individuals, mostly less than 40 years of age(6, 7). Therefore, these alarming reported data elucidate the need to find new efficient therapeutic options to GC, that remains a major challenge and concern(8).

In the last two decades, targeted therapies using TKI has been reported as an emerging promising approach in the clinical setting(9). Efforts to improve drug delivery reducing its side effects, have led to the development of functional nanodelivery systems as they increase the potential for an effective delivery of the drug in cancer therapy to cancer cells(10, 11). To improve the specificity of the NPs, ligands, such antibodies, might be attached to the surface of NPs by target glycans aberrantly expressed by cancer cells(12, 13).

The aberrant biosynthesis of glycans is commonly involved in fundamental molecular and cell biology processes associated with cancer. This leads to the display of specific cancer-associated glycan structures with important roles in tumor progression, invasion, angiogenesis and metastization(14, 15). Moreover, the use of cancer-exclusive glycans as targets for drug delivery has been shown to be an effective strategy for cell-selective drug delivery, as we described in our overview **Appendix I**(13).

As described in **Chapter 1**, STn is a truncated O-glycan aberrantly expressed in cancer, which is expressed by more than 80% of human carcinomas, including GC, contrarily to normal tissues(16-18). This antigen has associated with poor prognosis and resistance to therapy(17-19). Considering its high expression and association with GC malignant

features, the STn antigen has a higher potential as a target for therapy and particularly for a nanodelivery system using an antibody directed to STn.

In **Chapter 2**, the aim was to demonstrate the targeting and specific ability of polymeric NPs functionalized with an antibody directed to a cancer-exclusive glycan. Therefore, a targeted nanodelivery system was developed, to take advantage of the presence of an aberrant sialylated glycan, STn, present in mostly GC cells increasing the specificity of the treatment.

We demonstrated that we successfully produced the NPs, showing physicochemical properties suitable for an effective use. The surface of the polymeric NPs was functionalized with one of two antibodies: monoclonal antibody B72.3(20) or mouse IgG1. B72.3 antibody, responsible to do the targeting of the STn antigen expressed in the surface of MKN45 SimpleCell (SC) GC(21) cell line in this work. Contrarily MKN45 to Wild Type (WT), that did not express STn. In the other hand, the unspecific IgG1 antibody represents a non-targeted control when functionalized in our NPs. The quantification of conjugation efficiency (CE) of these antibodies to the NPs showed a high amount of antibody covalently bound. Previous studies report that could be an association between low CE with lower amounts of antibody(22-24). According with this, the high CE percentage observed in the developed NPs can be explained by the use of a high amount of antibody to surface functionalize these NPs, increasing the linkages between antibody and the NPs. In addition of the CE quantification, by ELISA, we analyzed qualitatively by ImmunoTEM where the efficient binding of the antibody B72.3 to the NP was indicated by the black dot in the micrograph.

Further, the capacity of this nanosystem loading a TKI molecule was verified and then characterized. We selected Lapatinib, a dual TKI (HER2 and EGFR)(25), known by its poor oral bioavailability, and poor water solubility, which results in poor and incomplete absorption from the gastrointestinal tract(26, 27). To overcome this obstacle, we developed an active targeting approach, with a stable and high-loaded polymeric formulation encapsulating Lapatinib in a nanosystem surface functionalized with an antibody directed to STn. The physicochemical characterization demonstrated a successful encapsulation with an association efficacy up to 50% of 1 mg of Lapatinib and a drug loading capacity of about 2.2%. Comparing this to others reports, for example Buss *et al.*, they report an association efficacy of 100% with a theoretical mass of 0.250 mg(28). We can conclude that our NP encapsulate higher amounts of drug, 0.467mg of Lapatinib.

Lastly, we performed a functional assay *in vitro* to evaluate the capacity of delivery of drug of these NPs to the GC cells with HER2 and EGFR overexpression. Upon incubation during different timepoints with 1 µg/mL of Lapatinib, NLlgG and NLB72.3, our results showed that NLB72.3 induces higher specific effect, decreasing the metabolic activity in GC cells expressing STn at 24h and 48h of incubation, demonstrating the capacity of release of these NPs. Regarding the effect of NLlgG, this could be due NPs sedimentation since this experiment was done at static conditions and consequently, we can observe unspecific release of Lapatinib.

In parallel within this study, we demonstrated the targeting and specific ability of these NPs quantitatively, by flow cytometry and qualitatively, by immunofluorescence. Both experiments showed higher functionalized-NPs internalization. Therefore, NPs functionalized with antibody against STn glycan only bind to GC cells expressing STn, while NPs functionalized with IgG1 did not bind to none of the GC cells. However, we showed in immunofluorescence assay, that in longer incubation periods of NLB72.3 with MKN45 WT, we begin to observe deposits of agglomerates of NPs in an unspecific way, once they deposit in places that we do not observe cells. This phenomenon could be due to sedimentation associated with static experiments.

In literature is described the pertinence of study NP-target cell interactions under flow conditions and how it is crucial to evaluate the cellular responses in physiological-like environments. In terms of NPs applicability, flow stimulation can critically affect the efficacy of the targeting or the drug delivery accumulation in the tumor region(29-31). Contrarily to under flow conditions, static culture promotes the sedimentation of NPs, supporting the local accumulation of NPs agglomerates, and resulting in unspecific binding to the target cells(32-34). Moreover, it has been reported that flow can affect cell-NP interactions and therefore the NP uptake via the cells, mimetizing the *in vivo* conditions(31, 35). Hence, the cell's response under shear stress should be considered for improved validation of the targeting specificity.

Here, we developed a perfusion system in a controlled microenvironment to better understand and tracking the interaction of the developed targeted polymeric NPs with glycoengineered cells expressing STn antigen, under a flow-induced shear stress. This new approach to study the effect of the NP interaction with cells under live flow conditions, provide the opportunity to track the NPs for a time period and for the analysis of their interaction with the target cell with all the detail.

We demonstrated that the targeting ability and specificity of the NPs is sustained under flow conditions as aggregates of FITC labeled NPs functionalized with B72.3 binding

only to the MKN45 SC cell model were observed. Although the NPs control functionalized with IgG did not bind to any cell model, we were able to see that both NPs labelled with FITC and functionalized with antibodies, appear to touch the cells in a non-specific way. Furthermore, the lack of adhesion of control NPs to cells demonstrate the specific targeting ability of this nanosystem to STn expressing cells. This proposed perfusion system is a novelty in the field since it allows to follow over time the interaction of NPs with the cells in live image acquisition in a controlled environment. Most of the studies published so far rely on the fixation of the cells after a certain incubation period with the NPs under flow conditions, around 24h or 36h, do not allowing to follow the interaction of the NPs along with time of incubation(29, 31, 36, 37).

In **Chapter 3** we took in consideration the successful and promisors results of our nanosystem. We aimed to validate this drug delivery system in *in vitro* multicellular tumor spheroids (MCTS) gastric cancer models and in *in vivo* models. Subsequently, we loaded polymeric NPs functionalized with an antibody anti-STn with Foretinib (FRT), an oral multikinase inhibitor targeting MET and RON receptors tyrosine kinases(38).

Herein, we first characterized the physicochemical properties of these NPs and we concluded that in general the NPs were once again successfully produced and are suitable to be used. The size of these formulations, ranged between 126 and 199 nm, being ideal for intravenous administration. This size of NPs have been reported to show be able to infiltrate through the tumor vasculature and this way could avoid the normal vasculature(39). The negative values of zeta potential and the round shape observed in the TEM analysis of the NPs also reinforced the successfully production of these formulations. Additionally, we obtained similar values of CE, demonstrating that the method that we implemented anteriorly using the covalent link between the amine group of the antibody and the carboxylic group from the PEG copolymer is effective. This is reinforced by the fact that the hydrophilic PEG block promotes the presentation of the carboxylic acid on the NP surface then will increase the number of immunoglobulins that were covalently linked(24, 40). Regarding the values of loading FRT in the NPs, we demonstrated that ranged between 84.1% – 90.2% of 1mg of AE. This quantification revealed to be very satisfactory, since previous studies using also polymeric NPs loaded with FRT exhibited an AE of 37% of 1 mg(41), demonstrating that our FRT loading system was highly efficient.

Similar with the results presented in **Chapter 2**, we validated and showed the specific targeting ability of our nanosystem in **Chapter 3**, using in *in vitro* MCTS. MCTS have

been previously shown to be a useful model since it could better mimic the tumor microenvironment and fill the gap between the 2D cell culture and *in vivo*(42, 43).

Moreover, in our evaluations we demonstrated that 1 µg/mL of drug loaded in the NFB72.3 was efficient to inactivate the RTKs MET and RON upon 24h of incubation. The cell viability assay confirmed the impact in cells expressing STn when incubated with NFB72.3. As expected, NFIgG NPs, that do not target STn antigen, did not show any effect in the activation of phospho MET and phospho RON in both GC cell models. Overall, these results support the specific targeting ability of our NPs, demonstrating their capacity for being uptake and promote the delivery of the drug to cells expressing STn. Moreover, these findings strongly sustain the safety and specificity of this novel nanosystem functionalized against STn and highlight its therapeutic potential *in vivo*.

The therapeutic effect *in vivo* demonstrated the capacity of intravenous administration of NFB72.3 reduce the MKN45 SC tumor growth compared to free FRT or NFIgG. With these results we reinforced the importance of loading FRT in a nanosystem directed to STn cancer associated antigen, when compared with free FRT. This is the first report demonstrating the specific delivery of FRT encapsulated in NPs functionalized with an antibody anti-STn and validated in gastric cancer mouse models. Nevertheless, some reports with free FRT have been performed in xenograft mice models with pancreatic(44), esophageal(45) and endometrial(46) cancer. In our work we demonstrated no effect on tumor volume reduction in the mice treated with free FRT when compared to NFB72.3, contrary to what was observed in previous reports(44-46). This could be associated to the fact that we used a different via of drug administration and also a different treatment scheme that includes less days of treatment. Importantly, we showed that when we loaded FRT in polymeric NPs that were functionalized with anti-STn antibody, we end up with a nanodelivery system able to decelerate the tumor growth.

Further analysis of our nanosystem also demonstrated that treatment with NFB72.3 consistently decreased tumor cell proliferation and necrosis area. In the other hand, the opposite was observed for the other groups of treatment, even for free FRT, which is in agreement with previous reports from Hassan *et al.* that described that esophageal adenocarcinoma xenograft tumors treated with free FRT did not decrease tumor cell proliferation(47).

Finally, we revealed the effect of our innovative therapy approach in MET and RON phosphorylation in the tumor tissues. We showed that NFB72.3 successfully inhibited the MET and RON phosphorylation, contrarily to the other groups. In literature, other

studies also demonstrated the same results when combined FRT with one more therapeutic drug(47, 48). Moreover, Kogata et al. described inactivation of phospho MET in endometrial cancer xenografts treated with 30 mg/kg(46). Despite the fact they used the same NP concentration, the scheme of administration was completely different, as it was administrated orally daily for 14 days(46). Hence, our data demonstrated that functionalizing FRT loaded NPs with antibodies against STn represents a new potential targeted therapy regimen in MET activated gastric cancers.

CONCLUSIONS

Gastric cancer remains a huge concern due to its high incidence and high mortality levels worldwide. The search for more effective targeted therapeutic with less side effects remains to be crucial to decrease this numbers.

In this study we developed a nanodelivery system functionalized with an antibody directed to STn antigen that allows to encapsulate tyrosine kinase inhibitors. We took advantage of the exclusive expression of STn in cancer and considered it as good target for drug delivery. Finally, we validated this formulation in two different studies, demonstrating their targeting ability and specificity and their capacity of drug release and tumor volume decrease.

In **Chapter 2** we showed that polymeric NPs functionalized with B72.3 antibody can successfully target STn glycan, highly expressed by cancer cells. Herein, is the first time that NPs functionalized with an anti-STn antibody were used for targeting STn expressing-cancer cells. Additionally, we evaluated the targeting ability of this nanosystem in live gastric cancer cells and under flow conditions. The live tracking of NPs consists in an innovative platform for *in vitro* experiments as it reproduces the physiological flow which may mimics the behavior and interaction of cells and NPs in these conditions. Thus, we propose that this microfluidic approach is a robust method to evaluate and characterize the targeting capacity of the NPs and the impact they can have on the targeting cells. Moreover, in contrast with studies developed under static conditions, we demonstrated that we were able to avoid the unspecific sedimentation of NPs aggregates under flow conditions, supporting the efficacy of this experiment. Finally, we also verified the capacity of these NPs to encapsulate Lapatinib and taken up specifically to STn expressing cells. The specificity of these novel NPs allied to successful encapsulation and release of a tyrosine kinase inhibitor demonstrates a great potential to be translated to *in vivo* studies of drug delivery.

In **Chapter 3** we have successfully developed an innovative nanosystem directed to cancer cells expressing STn and loaded with the tyrosine kinase inhibitor Foretinib. Our data showed to efficiently and specifically deliver the drug to cancer spheroids of gastric carcinoma cells expressing STn, demonstrating their impact in phospho MET and phospho RON activation and in cell viability of the cells. Interestingly, these NPs demonstrated the capacity to significantly decrease the tumor growth of gastric cancer mouse xenografts expressing STn, comparing to free FRT or non-targeted NPs. This proof-of-concept study highlights the potential of this novel nanodelivery approach directed to STn as a promising strategy to efficiently deliver drugs for GC treatment.

In conclusion, our data demonstrate that we developed a nanodelivery system able to encapsulate different tyrosine kinase inhibitors and deliver these drugs specifically directed to cancer cells expressing STn antigen. This allows the efficient delivery of the drug to cancer cells and to minimize therapeutic's side effects. Finally, and since STn is expressed in 80% of human carcinomas, this novel STn based NP approach might also be a suitable system for other drug delivery applications in different cancers settings.

FUTURE PERSPECTIVES

The present thesis has provided results and insights about two different tyrosine kinase inhibitors independently encapsulated in a nanodelivery system directed to STn antigen: Lapatinib and Foretinib. However, several questions regarding the impact of the combination of these two nanoparticles to treat gastric cancer remain unaddressed.

We have proposed a new approach to deliver each of these TKIs directly to GC cells expressing STn. Moreover, we have demonstrated in multicellular tumor spheroids and in xenograft mouse models of GC expressing STn the successful impact of treatment with NPs loaded with FRT and directed to this antigen. Additionally, we also showed the cytotoxic effect of NPs loaded with Lapatinib and functionalized with anti-STn antibody, in GC cells expressing STn antigen. To accomplish this, Hassan *et al.*, reported the combination effect of Lapatinib with Foretinib in HER2 and MET in esophageal adenocarcinoma(47). They demonstrate that this combination is more effective compared to the drugs individually. Finally, they suggest that this new therapy should be tested as a treatment option for HER2 positive patients with MET-overexpressing esophageal adenocarcinoma(47). Following this, it may be pertinent to test the combination of both NPs with Lapatinib and Foretinib in GC and in other cancers models that express the targeting receptors of these drugs.

Another interesting goal would be the encapsulation of these NPs with standard chemotherapeutics and this way we can minimize the side effects that are typically associated with these therapies and guarantee that the drug will be deliver only to cancer cells. Fernandes *et al.*, developed a delivery approach loaded with 5-fluorouracil or paclitaxel, typical chemotherapeutics, and directed to Sialyl Lewis A antigen(23). Other strategy can be the combination of these standard therapies with one of our novel NPs and validate this combination *in vivo*. Grojean *et al.*, also demonstrated the powerful effect of the combination of Foretinib and a NP loaded with paclitaxel, a standard chemotherapeutic, in gastric cancer models(48) .

As we mentioned before, the method defined to produce our NPs was the nanoprecipitation. This method is associated with high encapsulation yield of hydrophobic drugs(49), such Lapatinib or Foretinib. Thus, we think it would be interesting to encapsulate other hydrophobic drugs, highly toxic and with problems with poor solubility and poor permeability and difficulties to reach the target and evaluate them in the context of our nanodelivery system directed to STn. We hypothesized that we would minimize the side effects and, decrease the amount of drug needed to have a therapeutic effect due to the direct uptake of the drug to the cancer cells expressing STn.

STn antigen is a tumor associated glycan expressed in more than 80% of human carcinomas, and rarely expressed in normal tissues(16-18). Besides in GC, STn is highly expressed in breast, lung, ovarian, pancreatic and colorectal cancer. This emphasizes the importance of this antigen to be considered a good target for this nanodelivery approach. Moreover, reveals the several possibilities where this NP can be applied, since STn is overexpressed in so many tumors. Thus, the validation of these NPs in other carcinomas expressing STn is very appropriate to do in the future.

Lastly, it would be also relevant to perform the humanization of the antibody that we used in the process of functionalization of the NP, in order to test these NPs in humans in further clinical assays.

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CHAPTER 5

Appendix I

Appendix II

Appendix III

APPENDIX I

Glycans as Targets for Drug Delivery in Cancer

Francisca Diniz, Pedro Coelho, Henrique O. Duarte, Bruno Sarmiento, Celso A. Reis, and Joana Gomes

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Review

Glycans as Targets for Drug Delivery in Cancer

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Simple Summary: Alterations in glycosylation are frequently observed in cancer cells. Different strategies have been proposed to increase drug delivery to the tumor site in order to improve the therapeutic efficacy of anti-cancer drugs and avoid collateral cytotoxicity. The exploitation of drug delivery approaches directed to cancer-associated glycans has the potential to pave the way for better and more efficient personalized treatment practices. Such strategies taking advantage of aberrant cell surface glycosylation patterns enhance the targeting efficiency and optimize the delivery of clinically used drugs to cancer cells, with major potential for the clinical applications.

Abstract: Innovative strategies have been proposed to increase drug delivery to the tumor site and avoid cytotoxicity, improving the therapeutic efficacy of well-established anti-cancer drugs. Alterations in normal glycosylation processes are frequently observed in cancer cells and the resulting cell surface aberrant glycans can be used as direct molecular targets for drug delivery. In the present review, we address the development of strategies, such as monoclonal antibodies, antibody–drug conjugates and nanoparticles that specific and selectively target cancer-associated glycans in tumor cells. The use of nanoparticles for drug delivery encompasses novel applications in cancer therapy, including vaccines encapsulated in synthetic nanoparticles and specific nanoparticles that target glycoproteins or glycan-binding proteins. Here, we highlight their potential to enhance targeting approaches and to optimize the delivery of clinically approved drugs to the tumor microenvironment, paving the way for improved personalized treatment approaches with major potential importance for the pharmaceutical and clinical sectors.

Keywords: antibodies; cancer; drug delivery; glycans; glycosylation; nanoparticles



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1. Introduction

Cancer remains one of the deadliest diseases and a major burden worldwide. Over the last decades, early detection and effective treatment of cancer have been the main focus in the field. Indeed, major advances in the fields of molecular biology and the cellular basis of cancer development and progression have been made and contributed to a more comprehensive understanding of the disease [1].

Chemotherapy comprises powerful drugs against cancer that target key biological mechanisms leading to the killing of highly proliferative cells in body. However, the use and application of these drugs in cancer treatment have significant limitations, mostly

related to the dose-limiting toxicity, poor specificity towards tumor cells and the frequent emergence of drug resistance [2,3].

Targeting cancer cells is crucial to improve the efficiency of specific drugs, avoiding drug-induced toxicity in non-neoplastic tissues, but also to aid in the detection of cancer cells and, consequently, improving diagnostics. For this reason, the development of novel and more efficient strategies to specifically target malignant cells remains of major interest [4,5].

The current concept at the basis of precision oncology is that tumor-specific molecular abnormalities can be targeted with accurate, effective, and potentially less-toxic therapies. The first gateways for genomic accuracy in oncology were opened upon large preclinical work and primary discoveries of somatic, single-gene genomic abnormalities underlying oncogenic transformation that could be pharmacologically targeted. The resulting aberrantly expressed proteins can be selectively targeted by strategies using specific compounds and drugs. Recently, the use of aberrant glycans overexpressed by cancer cells have emerged as potential molecular candidates for the improvement of cancer targeted therapies [6,7].

2. Glycosylation in Cancer

Glycosylation is a highly regulated enzymatic process comprising the addition of an initial monosaccharide to a non-glycosyl aglycone, usually a protein or a lipid. The initial sugar is further elongated with additional monosaccharides, which are linked covalently through glycosidic bonds, forming oligosaccharides or polysaccharides. Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus, where the highly orchestrated and organized activity of both glycosyltransferases and glycosidases produces a vast repertoire of structurally complex and functionally diverse carbohydrate structure, or glycans [8] (Figure 1).

In protein glycosylation, glycans are typically attached via glycosidic linkages to the nitrogen atom on the side chain of asparagine (N-glycans) [9] or the oxygen atom on the side chain of threonine or serine (O-glycans) [10]. Other important type of glycoconjugates are glycosphingolipids (GSLs), cell-surface glycolipids formed by a hydrophobic ceramide core and a hydrophilic residue [11]. On the other hand, proteoglycans are glycoconjugates with one or more glycosaminoglycan (GAG) chains, such as chondroitin sulfate, heparan sulfate and keratan sulfate, attached to a protein backbone [12].

Glycans are produced in a non-templated way and their synthesis is controlled by substrate availability, gene transcription levels, tissue-specific expression patterns of glycosyltransferases and glycosidases, enzyme location within the compartments of the secretory pathway, chaperone activity, donor substrate availability, and modifications in the tertiary conformation of acceptor peptides [13,14].

Cell surface glycoconjugates form the glycocalyx, a dense gel-like protective barrier that shields the plasma membrane from physical stress while also shaping various aspects of cell surface dynamics. Furthermore, cell surface glycoconjugates are key regulators of various molecular and cellular processes, including intracellular trafficking, cell–cell interactions, cell–matrix adhesion, as well as molecular signaling, immune regulation, host-pathogen recognition and malignant transformation [13] (Figure 1).

In cancer, aberrant expression and mislocalization of glycosyltransferases and glycosidases, as well as imbalances affecting the availability of sugar nucleotide donors, lead to the emergence of aberrant glycosylation patterns (Figure 1). These cancer-derived glycan structures are key players in tumor biology, as they actively support to the various neoplastic characteristics of malignant cells [15]. In fact, it has been extensively demonstrated that cancer-associated glycans mediate the crosstalk between cancer cells and their surrounding microenvironment, contributing for the acquisition of cancer hallmarks, such as sustained proliferative signaling, resistance to cell death, immune system evasion, angiogenesis, invasion and metastization [13] (Figure 1). Receptor tyrosine kinases (RTKs) and their downstream signaling cascades actively drive neoplastic transformation. Moreover, sev-

eral studies have demonstrated that the altered glycosylation of different RTKs (such as EGFR, ErbB2, Met, and RON) leads to their hyperactivation, which, in turn, promotes RTK-dependent malignant growth and phenotypically aggressive tumors [16–20].

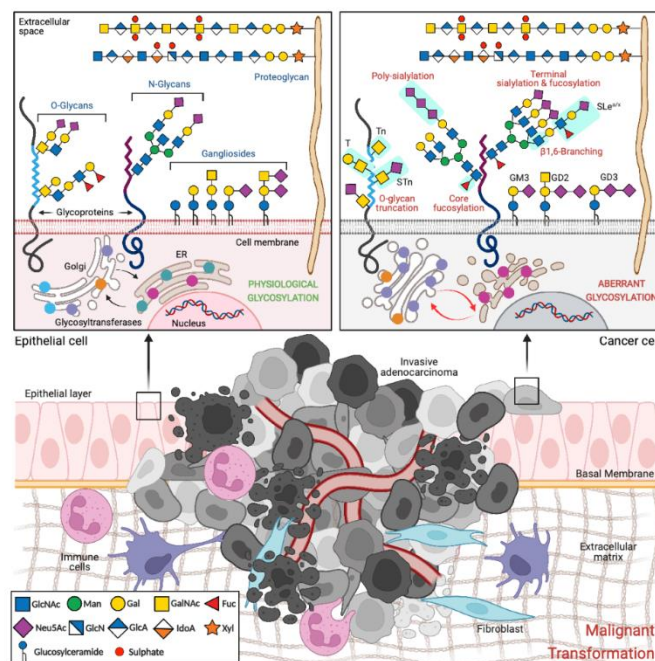


Figure 1. Glycosylation alterations during malignant transformation. Abbreviations: ER—endoplasmic reticulum; Fuc—fucose; Gal—galactose; GlcN—glucosamine; GlcA—glucuronic acid; GlcNAc—N-acetylglucosamine; GalNAc—N-acetylgalactosamine; IdoA—iduronic acid; Man—mannose; Neu5Ac—N-acetylneuraminic acid; STn—sialyl Tn; SLe^{a/x}—sialyl Lewis a/x; T—Thomsen–Friedenreich antigen; Xyl—xylose.

2.1. Glycans with Specific Expression in Cancer

Aberrant glycosylation in cancer leads to the biosynthesis of tumor-associated glycans that underpin several oncogenic features leading to tumor onset and progression. Altered glycosylation encompasses the synthesis of prematurely truncated O-glycans, such as the single monosaccharide N-acetylgalactosamine (GalNAc; Tn antigen), sialyl Tn (STn) and the Thomsen–Friedenreich (T) antigen, as well as an increase in N-glycan β 1,6-branching, terminal sialylation, including the biosynthesis of polysialic acid-containing structures, and both core and antennae-linked fucosylation. These alterations support the enrichment of cell surface glycoconjugates with sialofucosylated Lewis antigens (sialyl Lewis x (SLe^x) and sialyl Lewis a (SLe^a)) [13] (Figure 1). These tumor-associated glycan structures can be present on the surface of cancer cells, but they can be also secreted or shed into the circulation and, thus, serve as potential biomarkers of disease (such as SLe^a serologically detected by CA19-9, STn detected by CA72-4, MUC16 detected by CA125 and MUC1 detected by CA15-3) [13]. Due to their unique characteristics and highly restricted expression in cancer, these tumor-associated glycans are emerging as promising targets in the context of targeted therapies.

2.1.1. Truncated O-Glycans

O-glycosylation produces mature, elongated, and branched O-glycan chains, often terminally modified with sialic acids or N-acetylneuraminic acid (Neu5Ac) through the action of sialyltransferases. A set of 20 GalNAc-transferases (encoded by *GALNTs*) initiates the biosynthesis of O-GalNAc glycan chains [21]. This family of enzymes transfers GalNAc to Ser/Thr residues of proteins, forming the Tn antigen (GalNAc α 1-O-Ser/Thr). Subsequently, the Tn antigen is modified by different glycosyltransferases, forming complex and mature O-glycans. Glycosyltransferase core 1 GalNAc β 1,3-galactosyltransferase 1 (C1GalT1) is responsible for adding galactose (Gal) to the Tn antigen, forming the core 1 O-glycan structure or the T antigen (Gal β 1-3GalNAc α 1-O-Ser/Thr). Core 1 β 3-Gal-T specific molecular chaperone (COSMC), localized in the ER, is necessary for the activation of C1GalT1 in the Golgi apparatus by preventing its misfolding, aggregation and proteasomal degradation [22]. Core 1 structures can be further modified by core 2 β 1,6-N-acetylglucosaminyltransferase (C2GnT), which adds N-acetylglucosamine (GlcNAc) to the T antigen, forming core 2 O-glycans. These structures can then be subsequently modified by different glycosyltransferases, including sialyl- and fucosyltransferases, forming increasingly complex structures [10].

In cancer, alterations affecting the glycosylation machinery trigger the expression of immature and truncated O-glycans, such as Tn antigen, and its sialylated form, STn (NeuAc α 2-6-GalNAc α 1-O-Ser/Thr) [13]. In non-transformed cells, early-acting enzymes responsible for core O-glycan biosynthesis, such as C1GalT1 and C2GnT, are localized in cis- and medial-Golgi, and late-acting enzymes, such as sialyltransferases, are localized in the trans-Golgi. In the cancer setting, however, the overexpression of sialyltransferases leads to their aberrant distribution across all of the Golgi cisternae, where they will compete with early acting-enzymes and contribute to the emergence of immature forms of O-glycan chains. One example is the overexpression of the α -GalNAc α 2,6-sialyltransferase I (ST6GalNAc1) that leads to the premature addition of sialic acid to form the STn antigen [23,24].

While STn expression is neglectable in normal tissues, high levels of this short O-glycan antigen have been widely reported across different epithelial-derived tumors, such as gastric, pancreatic, colorectal, and breast, and are associated with poor patient survival [25]. The mechanisms involved in STn biosynthesis include loss of C1GalT1 expression, mutations and hypermethylation of COSMC, and overexpression of ST6GalNAc1 [24,26]. STn overexpression in gastrointestinal cancer cells leads to a more aggressive phenotype, supporting the epithelial-to-mesenchymal transition (EMT) process and reducing cell-cell adhesion capacity through the loss of E-cadherin expression [27–29]. Furthermore, STn-overexpressing cells are more capable of degrading and invading the surrounding extracellular matrix (ECM), being associated with poor clinical outcome and chemotherapy resistance [27–30].

In cancer, several glycoproteins are overexpressed and aberrantly glycosylated, carrying tumor-associated glycans underpinning tumor growth and metastization [15]. CD44, a transmembrane receptor for hyaluronic acid (HA) and other ECM molecules, is a well-known glycoprotein, often overexpressed in several cancers and reported as a major carrier of truncated O-glycans in gastric cancer cells. The presence of these truncated O-glycans impact CD44 binding to HA, while also promoting hyperactivation of RON receptor [31]. Another prominent glycoprotein in the context of cancer is MUC1, a highly glycosylated transmembrane mucin, that has also been reported to be a carrier of STn in multiple cancer types, and whose overexpression has been linked to tumor growth and metastization [32].

2.1.2. Sialylated Glycans

One of the main terminal modifications of glycans is the addition of a negatively charged acid (Neu5Ac) to glycoproteins and GSLs, contributing to their biophysical and biological functions. Sialylation of glycoproteins plays key roles in the blood and lymphatic vasculature, being essential for cell-cell and glycoprotein-protein interactions [33–35]. The SLe^x and SLe^a isomers mediate, under physiological conditions, the binding of circulating

leukocytes to cell surface selectins expressed on activated endothelial cells during immune responses [36]. A frequent alteration that occurs in several cancers is the increased expression of SLe^x and SLe^a. As such, tumor cells bind to vascular endothelial selectins, enhancing their migration and extravasation [37,38]. ST3Gal3, ST3Gal4 and ST3Gal6 are upregulated in different cancer types and drive the expression of SLe^x. In gastric cancer, high expression of ST3Gal4 is associated with an increased capacity of tumor cells to invade and metastasize. In addition, SLe^x expression in tumor cells has been reported to promote proliferation, motility, angiogenesis, while protecting tumor cells from apoptosis [20].

The glycoprotein carcinoembryonic antigen (CEA) is an oncofetal antigen expressed in the developing fetus and in epithelial malignancies, but only in neglectable amounts in normal adult human tissues. In gastric carcinoma, CEA was described as the main carrier of SLe^x, and this glycoform was associated with a more aggressive phenotype [39].

Apart from Neu5Ac, others non-human members of sialic acid family, named N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), although deficient or less abundant than Neu5Ac in humans, have also been detected with elevated levels in human cancers [40–42].

2.2. Glycan-Binding Proteins

Immune cells express glycan-binding protein (GBP) receptors, such as galectins, C-type lectin receptors (e.g., selectins) and sialic acid-binding immunoglobulin-like lectins (Siglecs) that, upon the recognition of specific glycan ligands, activate tolerogenic or immunogenic signaling pathways [43,44]. The Siglec family consists of 15 transmembrane proteins expressed by most immune cells, and can be classified into two groups: the CD33-related Siglecs with high sequence identity (Siglec-3, -5, -6, -7, -8, -9, -10, -11) and the structurally conserved Siglecs (Siglec-1, -2, -4 and -15) [45]. Recognition of tumor-associated glycans by GBPs expressed on immune cells leads to production of anti-inflammatory cytokines, inhibits natural killer (NK) cell-mediated cytotoxicity, and activates immunosuppressive regulatory T cells [45]. For example, Siglecs are differentially expressed by immune cells and are capable of recognizing sialic acid motifs in a linkage-specific pattern (α 2,3, α 2,6 or α 2,8) [43,46]. Upon binding to sialic acids, Siglecs' intracellular immunoreceptor tyrosine-based inhibition motifs induce strong inhibitory signals that suppress the immune response [46,47].

3. Specific Targeting of Cancer Cells

The timely and accurate diagnosis and treatment of cancer requires the unequivocal in vivo detection and targeting of neoplastic cells. Indeed, the process of malignant transformation of healthy cells is underpinned by a plethora of molecular aberrations occurring at the genomic, epigenomic and post-translational levels. Such oncogenic events, many of which are therapeutically actionable, trigger the emergence of an array of both cell surface and intracellular tumor-specific neoantigens, whose expression is undetectable or neglectable in non-transformed cells. The highly restricted expression of such neoantigens within the cancer cell population makes them suitable molecular targets for antibody-based recognition, as well as appealing candidates for the development of both targeted therapeutic strategies and imaging systems for disease diagnosis. Since most therapeutic strategies for targeted drug delivery rely on the recognition of cell surface-residing molecules, the ideal target antigen should be broadly expressed at the membrane of tumor cells, while absent from the cell surface of healthy cells. Thus, the expression of aberrantly glycosylated forms of cell surface glycoconjugates in cancer cells makes these molecular entities appealing targets for highly selective and specific drug delivery systems (reviewed in [6,13]) (Figure 2).

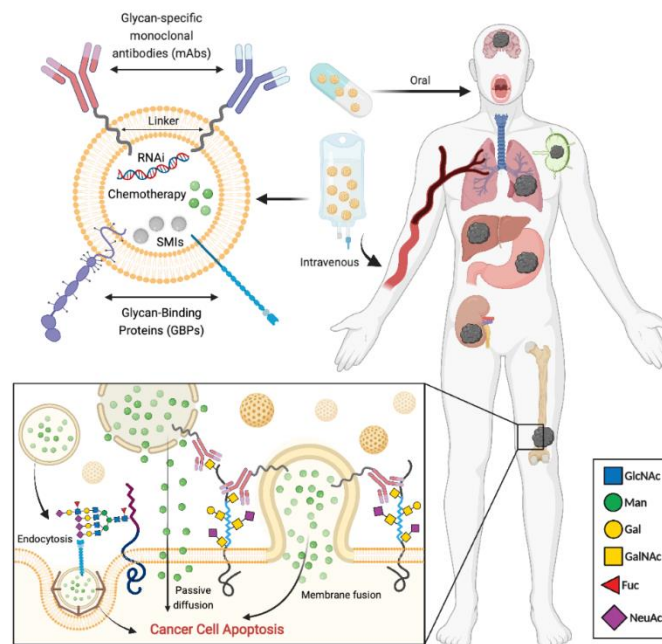


Figure 2. Strategies based on targeting cancer-associated glycans for selective and specific drug delivery systems.

3.1. Monoclonal Antibodies

Monoclonal antibodies (mAbs) targeting antigens that are either unique to (neoantigens) or overexpressed by malignant tissues trigger cancer cell death through a variety of effector mechanisms [48]. These include the blocking of receptor-induced proliferative signaling, the activation of anti-tumor immune responses, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP). Antibody-triggered immune-mediated responses require the engagement of the fragment crystallizable (Fc) region of the therapeutic mAb with multiple Fc receptors (FcRs) expressed at the surface of distinct immune cell populations, including NK cells, neutrophils, monocytes, dendritic cells and eosinophils [49]. Such interaction triggers the activation of a repertoire of specialized functions aiming at eliminating the cell to which the mAb is bound. Importantly, the immunoglobulin subclass further determines the capacity of a given therapeutic mAb to elicit immune-mediated effector responses. For instance, mAbs belonging to the IgG1 and IgG3 subclasses are able to induce the activation of ADCC and CDC, while IgG2 and IgG4 are not [50]. Several clinically approved mAbs target key immune checkpoints, such as the ones mediated by CTLA-4 and PD-1/PD-L1, and aim at blocking inhibitory self-tolerance signals. Such immunotherapies counteract the establishment of an immunosuppressive microenvironment and, thus, the escape of tumor cells to immune surveillance [51].

The advent of the hybridoma technology nearly 50 years ago allowed the generation of a virtually unlimited catalog of highly specific mAbs for the reliable detection of protein-, lipid- and glycan-based tumor-specific antigens. In the hybridoma system, during the immunization of a host organism, naive antibody-secreting B-cells are challenged with the purified target antigen [52,53]. Subsequently, activated cell clones are fused with

myeloma cells for immortalization, generating a hybrid, clonal and antibody-producing cell line, or hybridoma. Additional phage display technology has also been applied for the production of antibodies [54,55]. The resulting mAbs can then undergo chimerization or, more recently, full humanization to reduce their immunogenicity, and are subsequently used as targeted therapeutic agents [56]. In the context of biomarker-guided precision oncology, the detection of the target antigen in individual tumor specimens is warranted to determine patient eligibility for mAb-based therapeutic regimens. Such is the case of clinically approved therapeutic mAbs trastuzumab and pembrolizumab/nivolumab, used in the treatment of multiple ErbB2- and PD-L1-expressing solid tumors, respectively [57].

Over the past three decades, more than 20 therapeutic mAbs have been granted Food and Drug Administration (FDA) approval for the treatment of both solid and circulating tumors, most of which belong to the IgG1 subclass [49]. Most mAbs approved in the clinical setting target oncogenic RTKs and their cognate ligands, whose overexpression actively upregulates transcriptomic networks sustaining cancer cell proliferative signaling and angiogenic growth. Amongst the most prominent molecular targets are: two members of the epidermal growth factor receptor family (EGFR and ErbB2); the vascular endothelial growth factor receptors and ligands (VEGFR2 and VEGF); platelet-derived growth factor receptors (PDGFR α); and key immune checkpoint regulators (PD-1/PD-L1, CTLA-4). Some mAbs directed at cancer-derived glycopeptides and other glycan-based antigens have shown promising selectivity towards cancer cells and potent cytotoxicity. These include mAbs generated against Tn/STn-modified MUC1 and MUC16 glycopeptides, and the clinically approved dinutuximab targeting the GD2 GSL, approved in the clinical setting as a second-line therapeutic agent for the treatment of high-risk neuroblastoma [58–61].

Although the clinical implementation of such compounds has proven successful in the treatment of cancer, both innate and acquired molecular resistance continue to pose a significant challenge to the efficacy of therapeutic mAbs. These include the downregulation of the cell surface molecule targeted by the mAb [62], the activation of bypass signaling circuits that circumvent mAb-mediated receptor blockade [63], and the acquisition of mutations in the target molecule that either prevent mAb binding or significantly reduce target affinity [64,65]. Furthermore, due to the poor vascular irrigation and impaired lymphatic drainage of most solid tumors, efficient extravasation, tissue penetration and homogeneous distribution of systemically administered mAbs at the target tumor site have proven challenging to achieve [66]. Furthermore, glycans have a major impact on target epitope recognition for cancer diagnosis and therapeutic stratification [6,67,68]. Indeed, glycan-mediated epitope masking hampers the *in situ* detection of therapeutic targets such as the PD-L1 immune checkpoint [68]. Enzymatic glycan removal improves PD-L1 detection in cancer patient tissues and predicts the clinical benefit from immunotherapeutic regimens [69].

The stability, immunogenicity and biological activity of therapeutic mAbs can be further optimized through glycoengineering, i.e., by rigorously controlling the glycosylation status of conserved sites at the Fc region [70]. Indeed, subtle changes in the cell culture and downstream processing procedures significantly influence the glycosylation pattern of produced mAbs, which, in turn, crucially impacts their structural and functional properties. MABs of the IgG class harbor a conserved site for N-linked glycosylation at the Asn297. For instance, the removal of core fucose from this glycosylation site, or fucosylation, has been shown to increase the binding affinity of IgG1 mAbs to their cognate Fc γ RIIIa receptor through reduction of steric hindrance [71,72]. The decoration of complex N-glycan structures with terminal Neu5Ac motifs, on the other hand, inhibits the binding affinity of mAbs to the Fc γ RIIIa receptor and, consequently, dampens the activation of ADCC responses [73]. Terminal galactosylation of glycan chains decorating Asn297 has been reported to induce CDC-based immune responses [74]. Additionally, specific glycosylation profiles of therapeutic mAbs crucially govern their pharmacokinetic properties. For instance, the modification of Asn297 with oligomannosidic, or high-mannose, N-glycan species confers therapeutic mAbs faster clearance rates [75].

With the exceptions of mAbs targeting CD20, ErbB2 and EGFR, most therapeutic mAbs approved in the clinical setting bear modest cytotoxic activity on their own. Regardless, given their high specificity to tumor-derived antigens, mAbs represent extremely versatile platforms for the targeted delivery of cytotoxic drugs to the tumor site, such as conventional chemotherapeutic agents (antibody-drug conjugates (ADCs)), while reducing the occurrence of off-target toxicity. More recently, therapeutic antibodies have been engineered to bind to more than just two structurally identical epitopes (multi-valency), as well as to structurally distinct epitopes (multi-specificity) [76]. Such is the example of bi-specific T cell Engagers (BiTEs), which aim at stimulating anti-tumor immune-mediated synapses by simultaneously targeting a tumor antigen and the activating T cell receptor CD3 [77]. Other bi-specific mAb formulations aim at effectively neutralizing RTK-induced oncogenic signaling, by blocking cell surface activation of multiple target receptors [78].

3.2. Antibody-Drug Conjugates

The specificity of mAbs towards their cognate antigen can be advantageous in the targeted delivery of non-specific cytotoxic agents to cancer cells, whilst avoiding healthy tissues. Such ADC formulations currently represent the fastest growing drug class in oncology. These therapeutic formulations are composed of three main elements: a cytotoxic drug (also known as cytotoxic payload), a mAb and a flexible linker [79]. The mechanistic principle underlying this strategy is based on the highly specific recognition of a cellular surface antigen, whose expression is highly restricted to the cancer cell population, by the mAb moiety, and the subsequent delivery of the cytotoxic payload to the tumor tissues. Such systems can, therefore, improve the efficacy of chemotherapy and reduce systemic exposure to the drug's toxicity and side effects [80]. ADCs exert cytotoxicity through two distinct mechanisms. The primary mechanism consists in the cellular internalization of the ADC, followed by the intracellular release of the payload after linker cleavage. The secondary mechanism takes place when the payload or payload-linker permeates adjacent cells, including those with absent expression of the target antigen [79,81].

Until now, nine ADCs have been approved by FDA (Table 1). However, only five have so far received regulatory approval by both the FDA and the European Medicines Agency (EMA) for the treatment of cancer: brentuximab-vedotin, trastuzumab-emtansine (TDM-1), inotuzumab-ozogamicin, gemtuzumab-ozogamicin, and polatuzumab-vedotin. Moreover, dozens of other ADCs are currently under preclinical and clinical stages of development [79,80]. ADCs are thus becoming an important and viable strategy for targeting cancer cells and deliver highly cytotoxic drugs in a highly targeted manner, while protecting the integrity of non-malignant tissues.

The flexible linker of ADCs plays two important roles: ensuring that the cytotoxic payload is firmly connected to the antibody, and allowing its efficient release at the target tumor site [79,80]. It is essential that linkers sustain the stability of the ADC during blood circulation to guarantee its integrity until the target tumor site is reached. Linkers can be classified into two groups: cleavable and non-cleavable linkers. The cleavage of some linkers is dependent on physiological conditions, such as pH of the recipient cell. On the other hand, non-cleavable linkers confer the ADC improved stability while in circulation, increasing the ADC systemic half-life and decreasing off-target toxicity [80]. Of the nine currently FDA-approved ADCs (Table 1), two contain non-cleavable linkers: TDM-1 [82] (indicated for ErbB2-positive metastatic breast cancer) and belantamab-mafodotin [83] (approved for relapsed or refractory multiple myeloma).

Early developed ADCs were design to carry traditional chemotherapeutic drugs (methotrexate, doxorubicin and vinca alkaloids). However, most of these formulations had compromised efficiency which required extremely high dosing [79,80]. Currently, the most widely used payloads, such as auristatins, calicheamicins, maytansinoids and camptothecin analogues, depict higher potency at sub-nanomolar concentrations. The most commonly used payloads target either DNA replication or tubulins. Considering the nine FDA-approved ADCs, only two of them do not target DNA or tubulins: trastuzumab-

deruxtecan [84] (approved for metastatic ErbB2-positive breast cancer) and sacituzumab govitecan [85] (indicated for triple-negative breast cancer). The payload target of these two ADCs is topoisomerase I (TOPO1). The strategy of TOPO1 inhibitors, in patients with breast cancer, is being used in advanced stages, contrarily to microtubule-targeting agents which are more broadly used in early lines of treatment. This approach makes the treated tumor more susceptible to the cytotoxicity induced by TOPO1 inhibitors and avoids the emergence of therapeutic resistance [79].

Table 1. Panel of antibody-drug conjugates (ADCs) approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

ADC Formulation	Target Antigen	Antibody	Approved Clinical Application ¹	Year of Approval	Approving Regulatory Entity
Brentuximab-vedotin (SGN-35, Adcetris)	CD30	Chimeric IgG1	Hodgkin's lymphoma, ALCL, PTCL, MF	2011/2012	FDA and EMA
Trastuzumab-emtansine (T-DM1, Kadcyla)	ErbB2	Humanized IgG1	ErbB2-positive metastatic breast cancer	2013	FDA and EMA
Inotuzumab-ozogamicin (Besponsa)	CD22	Recombinant humanized IgG4	B cell precursor ALL	2017	FDA and EMA
Gemtuzumab-ozogamicin (Mylotarg) ²	CD33	Humanized IgG4	CD33-positive AML	2017/2018	FDA and EMA
Polatuzumab-vedotin (Polivy)	CD79	Humanized IgG1	DLBCL	2019/2020	FDA and EMA
Enfortumab-vedotin (ASG-22ME, Padcev)	Nectin-4	Human IgG1	Advanced urothelial cancer	2019	FDA
Trastuzumab-deruxtecan (DS-8201a, Enhertu)	ErbB2	Humanized IgG1	Metastatic ErbB2-positive breast cancer	2019	FDA
Sacituzumab-govitecan (IMMU-132, Trodelvy)	TROP2	Humanized IgG1	Triple-negative breast cancer	2020	FDA
Belantamab mafodotin (GSK2857916, Blenrep)	BCMA	Humanized IgG1	Relapsed or refractory multiple myeloma	2020 (orphan drug since 2017 by the EMA)	FDA

¹ ALCL, anaplastic large cell lymphoma; PTCL, peripheral T cell lymphoma; MF, mycosis fungoides; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; DLBCL, diffuse large B cell lymphoma. ² Gemtuzumab-ozogamicin was approved in 2000 and withdrawn from the market in 2010, then was re-approved in 2017 by the FDA.

The efficient and successful delivery of ADCs to cancer cells relies on the molecular target. Ideally, the selected target antigen should be widely expressed on the cancer cell surface to enable binding of the circulating ADC and absent or poorly expressed in healthy tissues to minimize off-target toxicity. Nevertheless, the efficacy of ADCs is also dependent of their specific binding affinity or internalization rate [79,80]. The molecular targets of four out of nine approved ADCs are homogeneously expressed within the target cancer tissues: inotuzumab ozogamicin (indicated as B cell precursor in ALL and targeting CD22) [86], gemtuzumab ozogamicin (approved for CD33-positive AML and targeting CD33) [87], brentuximab vedotin (indicated in Hodgkin's lymphoma, ALCL, PTCL and MF and targeting CD30) [88], and polatuzumab vedotin (indicated in DLBCL and targeting CD79) [89]. ErbB2 constitutes a cell surface RTK widely expressed in epithelial-derived tumors. Thus, the use of FDA-approved ADCs for the treatment of ErbB2-addicted solid tumors, which include TDM-1 [82] and trastuzumab-deruxtecan [84], is solely indicated in tumors with confirmed ErbB2 expression.

Most malignant tumors are able to escape immune surveillance and immune-mediated destruction. The cell surface presentation of Neu5Ac by tumor cells constitutes one of the main mechanisms underlying the capacity of tumor cells to evade immune-mediated responses. Thus, strategies targeting tumor-associated sialosides to potentiate anti-cancer immune response constitute promising therapeutic avenues. In line with this, Xiao et al. developed a trastuzumab-sialidase conjugate that selectively removes Neu5Ac moieties from ErbB2-expressing tumor cells, as well as ErbB2-negative neighboring cells, immunoediting their glycocalyx and increasing tumor cell vulnerability to ADCC [90].

3.3. Nanoparticles: Application of Nanotherapeutics in Cancer Therapy

In recent years, nanoparticles (NPs) have been extensively explored in biomedical research. Nanomedicines have several advantages over conventional cancer therapies. Firstly, they offer greater efficiency in early detection, diagnosis, imaging, and treatment of cancers [91]. Secondly, nanoscale engineering allows the production of multifunctional and “smart” NPs with extraordinary capacities, such as the ability to cross biological barriers, to target specific cells, and to control the drug release [5]. NPs have been widely used in the cancer field due to their high sensitivity for diagnostic-based imaging but also due to their use as alternative tools for targeted drug delivery with reduced systemic toxicity. The progress and improvements of nanosystems make them promising tools in clinical oncology [92].

The most prominent feature of therapeutic NPs is their capacity to efficiently encapsulate drugs with poor solubility, which they then efficiently deliver to the target tissue, while minimizing off-target toxicity [92] (Figure 2). Indeed, specific characteristics of NPs determine nanocarrier drug delivery efficiency and biodistribution in the body [5]. The most efficient way for a NP to systematically deliver a drug to the target tumor site is by remaining in circulation for a prolonged period. To do so, the nanodrug delivery efficiency can be controlled by modulating NP size and surface physicochemical characteristics [5].

Despite its known toxicity to non-cancer tissues, chemotherapy remains the most widely used therapeutic strategy in the cancer clinical setting. Doxorubicin, 5-fluorouracil and paclitaxel are known as potent anti-cancer drugs for the treatment of multiple cancers. Although these drugs efficiently impair cancer cell proliferation and growth, their systemic side effects and toxicity are also observed in healthy tissues, especially those characterized by significant cellular proliferation and turnover. For these reasons, several potent anti-tumor agents have not progressed into further clinical development [92]. In this context, NPs may represent a valuable alternative to reduce a drug’s off-target adverse effects, by restricting the release of the chemotherapeutic agents to the target tumor site. Nevertheless, there are several challenges related to the use of nanotherapeutic products, such as unintended NP accumulation, as well as pharmacokinetic, physicochemical and safety profiles [93–95].

Decorating Nanoparticles with Monoclonal Antibodies for Selective and Efficient Cancer Cell Targeting

The type of target tissue is determinant when designing nanodrugs. Nanocarriers can deliver drugs to tumors by two different mechanisms: passive and active targeting.

Passive targeting is accomplished by delivering NPs to defined organs via two different mechanisms, such as the reticuloendothelial system, or the enhanced permeability and retention (EPR) system [93,96]. However, these strategies have some limitations inherent to NP physicochemical properties and the pathophysiological features of cancer tissues. Firstly, the size and surface properties of NPs need to be highly controlled to avoid both renal clearance and uptake by the mononuclear phagocyte system, both of which reduce their circulation half-life. Besides, strict control over the NP size is also important to avoid NP accumulation by the EPR effect at the tumor site, through both convection and diffusion processes. This is expected to affect NPs in the 100–400 nm diameter range [93,95,96]. Furthermore, several chemotherapeutic drugs may induce multiple-drug resistance, which

makes anti-cancer therapy become inefficient due to the resistance of cancer cells. This is a consequence of the ineffective drug delivery mechanism that lacks controlled diffusion into the tumor, accumulating NPs improperly in tissues and preventing their prolonged circulation in the bloodstream [5,93]. The passive strategy is further limited in certain hypovascular tumors, which do not exhibit the retention effect and are supplied by vessels depicting heterogeneous permeability and distribution. Besides, the drug delivery process by passive targeting lacks control concerning the random nature of targeting and the inefficient drug diffusion into a tumor. Thus, NPs based on active targeting can help overcome these limitations [93].

Active targeting is based on the conjugation of NPs with molecules that specifically bind to a receptor whose expression is restricted to the target cell population, while depicting neglectable expression in normal cells [5]. The interaction between a ligand/mAb and its cognate receptor may induce NP internalization via receptor-mediated endocytosis and consequent intracellular drug release. Hence, non-specific interactions among NPs and normal cells will be minimized, resulting in decreased systemic side effects and increased cytotoxicity to tumor cells when applied to anti-cancer drug delivery [5,95,96]. Obviously, the success of ligand–receptor interaction depends on the amount of ligand/mAb at the NP surface, ligand orientation, and target receptor expression in the target cells. In this context, increasing the number of binding sites or generating novel binding sites are considered promising strategies to overcome the current limitations of active targeting [5,95,96]. Multiple ligands have been applied to functionalize NPs in the active targeting setting. Still, mAbs, mainly from IgG subtypes, remain the most widely used molecules to achieve active tumor targeting [93,97].

In the early stages of development, full antibodies were used as targeting ligands. Nevertheless, multiple challenges, including immunogenicity, poor stability, fast degradation and lower efficacy further encouraged the use of antibody fragments, which allow for higher loading capacities due to reduction in crowding, superior orientation of targeting ligands and reduced variability in NP diameter constant [93,97]. A NP functionalized with an engineered human Fab targeting the alternatively-spliced CD44v6 was developed, owing to its frequent overexpression in cancer tissues. Their nanodelivery system is characterized by specificity, optimal ligand orientation and low toxicity. Finally, they produced a NP capable of strongly binding to a CD44v6-derived peptide and, more crucially, to cells with endogenous CD44v6 expression, as opposed to CD44v6-negative cells [98,99].

4. Sweetening Precision Oncology with Glycan-Directed Nanoparticles

As previously mentioned, aberrant protein glycosylation constitutes an incontrovertible hallmark of the malignant transformation of human cells and tissues [6,13]. Moreover, the restricted expression of glycan-based cell surface neoantigens to cancer cells makes these molecular entities promising targets for selective anti-cancer drug delivery. Over the last decade, several efforts have been made to integrate nanotechnology and carbohydrate field for the targeted and controlled delivery of cancer therapeutic agents. Several studies have demonstrated the importance of using glycosylated NPs for therapeutic purposes (reviewed in [95,100]).

4.1. Vaccines Encapsulated in Synthetic Glycan-Targeting Nanoparticles with Glycan Targeting

Over the last decades, the development of vaccines has led to a significant expansion of the human life expectancy, with a positive impact on long-term protective immunity of the population [101]. The mechanism underlying therapeutic cancer vaccines relies on the modulation of the host's immune response against malignancies. Cancer cells are known to express a series of tumor-associated antigens that suppress the cytotoxic activity of T cell responses. Such is the case of STn- and Tn-decorated MUC1, which is overexpressed in several malignant tumors [95].

NPs can exert optimal actions both as delivery systems and as adjuvants, with the ability to extensively enhance immune responses while ensuring minimal toxicity [102]. A

study conducted by Liu et al. [103] produced an anti-tumor vaccine in which a MUC1 glycopeptide was used as the target tumor-associated immunogen, the α -galactosylceramide (α -GalCer) acted as an immune adjuvant, and gold-based NPs (AuNPs) as the multivalent carriers. The developed vaccine triggered a significant and durable antibody response, through specific binding of immunized serum to MUC1-expressing breast cancer cells. In another study, Cai et al. [104] developed a vaccine in which MUC1 glycopeptides were covalently linked to CD4 T-cell peptide epitopes and polyethylene glycol (PEG) combined with AuNPs. This formulation was able to promote antigen presentation by antigen presenting cells, modulating T-cell activation and MHC II-restricted T- and B-cell cooperation. Finally, Mocan et al. [105] demonstrated that AuNPs functionalized with a MUC1 protein fragment generated efficient anti-cancer responses via macrophage activation and polarization towards an inflammatory phenotype (M1). A polymerizable version of the Tn antigen was conjugated to AuNPs and was able to induce the strong and durable production of anti-Tn antibodies, demonstrating that the efficiency of glycan-directed vaccines is comparable to that of formulations targeting entirely peptidic antigens [106].

More recently, a novel antigen delivery system using MUC4, a glycoprotein highly overexpressed in pancreatic tumors, was decorated with the T antigen at different sites. Their synthetic vaccine targets antigen-presenting cells (APC) expressing dectin-1, a C-type lectin known to bind β 1,3-glucans. The obtained results showed that these particles induce strong in vivo immune responses, such as production of antibodies and the activation and proliferation of antigen-recognizing T-cells [107].

Some studies also focus on the new strategy based on the selective targets of the mannose receptor expressed in APC, such as dendritic cells and macrophages. It demonstrated a simultaneous enhancement of antigen-specific CD4+ and CD8+ T-cell responses by mannan-decorated poly(lactic-co-glycolic-acid) (PLGA) NPs with ovalbumin encapsulated, in comparison with non-decorated NPs [108]. In addition, drug-free mannosylated liposome NPs were also used to inhibit tumor growth by promoting the polarization of tumor-associated macrophages. It was shown that the induction of the M1 phenotype is associated to the increase of antitumor immune efficacy of immunomodulators [109].

4.2. Nanoparticle Strategies Using Glycoproteins

Several reports demonstrated the use of aptamers to target carbohydrate moieties, such as Neu5Ac, instead of mAbs [91,110–113]. Aptamers are synthetic oligonucleotides or peptide molecules with a small size, lower production cost, high specificity, and reduced immunogenicity [114]. A recent study [115] has developed an anti-MUC1 aptamer conjugated to a chitosan-based NP to co-deliver insulin-like growth factor receptor 1 (IGF-1R) siRNA and the docetaxel chemotherapeutic. This nanoformulation was conjugated with an anti-MUC1 aptamer and was shown to efficiently bind to MUC1-overexpressing metastatic breast cancer cells. Moreover, the developed NP was shown to be efficiently internalized by malignant cells and produce the desired downregulation of IGF-1R expression. Sayari et al. [111] have developed a similar nanocarrier system for the delivery of SN38, the active metabolite of irinotecan, to colon cancer cells. The poor solubility and high toxicity of SN38 have so far precluded its clinical use. However, the encapsulation and specific delivery of this compound to cancer cells may help overcome both these limitations. A MUC1-directed aptamer has been further conjugated with a mesoporous silica NP. The NP was loaded with epirubicin, a cytotoxic drug commonly used to treat breast cancer in the adjuvant setting, and depicted increased uptake and cytotoxicity in breast cancer cells [112]. Perepelyuk et al. evaluated the therapeutic efficacy of MUC1-aptamer functionalized hybrid NPs in lung tumor-bearing mice. This hybrid nanosystem was loaded miRNA-29b, a microRNA targeting DNA methyltransferases, and was shown to increase its specific delivery to the target cells and tissues [113]. Lastly, Yu et al. [110] also reported the potential of a MUC1 protein aptamer when as a targeting agent. Paclitaxel was loaded in aptamer-conjugated NPs of PLGA and validated in a MUC1-overexpressing breast cancer cells.

CD44 constitutes an additional appealing molecular candidate for anti-cancer targeted drug delivery [116]. One study has developed NPs coated with HA-appended PEG-PLGA polymers for the specific targeting of CD44-expressing triple-negative breast cancer cells. Such formulation, loaded with antisense miR-34a, depicted enhanced stability and potent in vivo toxicity [117]. A similar approach has been developed in the bladder cancer context, using CD44-targeting NPs loaded with anti-Bcl2 siRNAs [118]. Furthermore, PLGA-based NPs decorated with a Fab recognizing the CD44v6 variant highly expressed by gastrointestinal cancer cells have been developed [119].

Some other studies have employed methods to evaluate CEA, a cell surface glycoprotein overexpressed in several tumors, as a therapeutic target. A strategy was created for the targeting of triple-negative breast cancer using CEA-directed iron NPs, which depicted an enhanced tumor targeting capacity [120]. Additionally, CEA-targeting PLGA-PEG NPs loaded with paclitaxel have been demonstrated to selectively bind and trigger the death of CEA-expressing colorectal cancer cells [121].

4.3. Glycan-Targeting Nanoparticles

Targeting cancer-associated glycan epitopes is another promising strategy to achieve targeted drug delivery in cancer tissues. A commonly targeted glycan is SLe^a, due to its high expression during malignant transformation. In this context, functionalized PLGA NPs with a mAb targeting SLe^a and loaded with paclitaxel and 5-FU have been developed [122]. More recently, Palma-Chavez et al. developed and characterized a PLGA-based multi-stage delivery system for improved drug delivery to SLe^a-expressing inflamed vascular endothelium [123].

4.4. Nanoparticles Strategies Using Glycan-Binding Proteins

GBPs regulate the crosstalk between distinct sets of immune cell populations and tumor cells. By actively supporting the establishment of an immunosuppressive microenvironment, GBPs have emerged as promising targets of more efficient immunotherapies. Several studies have developed NPs functionalized with a mAb targeting Siglec-3 (CD33), which is abundantly expressed on the surface of acute myeloid leukaemia cells [124–127]. Furthermore, novel bi-specific liposomal-based NP T cell engagers (nanoTCEs) are conjugated with two mAbs targeting two distinct epitopes, one being a cancer antigen, and the other the Siglec-3 [124]. This formulation has shown to be efficient in the targeting and killing of AML cells, both in vitro and in vivo.

Another attractive target is Siglec-1 (CD169/sialoadhesin), due to its ubiquitous expression on macrophages. Chen et al. described a strategy using liposomal NPs with high binding affinity towards glycan-based ligands of Siglec-1 [128].

Several studies have been conducted using NPs functionalized with carbohydrate-recognizing lectins [129–133]. For instance, the mannose-binding concanavalin A was used to functionalize NPs and target sialic acids [129,133]. In addition, the conjugation of the wheat germ agglutinin to NPs loaded with anti-tumor chemotherapeutics has been shown to significantly enhance cancer cell-specific uptake, while minimizing off-target cytotoxicity [130]. Similar strategies have been employed for the targeting of cancer cells enriched in fucosylated antigens, such as Lewis x (Le^x), using the fucose-recognizing lectins *aleuria aurantia* lectin [131] and the lotus tetragonolobus lectin [132].

5. Future Perspectives

Development of therapeutic strategies using NPs directed at glycans constitutes an emerging and promising field. Recent studies demonstrate that NPs, with their unique properties, represent excellent platforms to be developed and applied in the new era of personalized target therapy. They not only improve the release and therapeutic efficacy of anti-cancer drugs at the target side, but also reduce cytotoxicity and a drug's off-target effects.

Several issues regarding NP formulation should be ensured in the future. The high level of consistency in the large-scale production, characterization, and reproducibility

of the engineered NPs must be guaranteed. Besides, a better understanding of the NP interaction with the immune system and their in vivo biodistribution is crucial which, together with their evaluation in preclinical studies using suitable animal models, will set the basis for future preliminary human trials. In addition, the NPs' fate and their biodegradability must be carefully evaluated, considering nanotoxicology issues.

The aberrant glycosylation landscape of tumor cells constitutes a major opportunity for developing advanced targeted therapies. The high prevalence of such glycosylation alterations expressed within the glycocalyx of tumor cells make them appealing target candidates for the antibody-based target delivery of NPs. Additionally, glycan recognizing molecules, such as antibodies, lectins, and other GBPs, unlock several potential strategies for clinical beneficial targeted therapeutics, ultimately improving patient clinical outcome.

Finally, the presently available nanoformulations targeting glycans have the potential to undergo improvements in order to ensure that these nanosystems could be efficiently translated into clinical practice.

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APPENDIX II

Analysis of the Effect of Increased α 2,3-Sialylation on RTK Activation in MKN45 Gastric Cancer Spheroids Treated with Crizotinib

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Article

Analysis of the Effect of Increased $\alpha 2,3$ -Sialylation on RTK Activation in MKN45 Gastric Cancer Spheroids Treated with Crizotinib

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Abstract: In the scenario of personalized medicine, targeted therapies are currently the focus of cancer drug development. These drugs can block the growth and spread of tumor cells by interfering with key molecules involved in malignancy, such as receptor tyrosine kinases (RTKs). MET and Recepteur d’Origine Nantais (RON), which are RTKs frequently overactivated in gastric cancer, are glycoprotein receptors whose activation have been shown to be modulated by the cellular glycosylation. In this work, we address the role of sialylation in gastric cancer therapy using an innovative 3D high-throughput cell culture methodology that mimics better the in vivo tumor features. We evaluate the response to targeted treatment of glycoengineered gastric cancer cell models overexpressing the sialyltransferases ST3GAL4 or ST3GAL6 by subjecting 3D spheroids to the tyrosine kinase inhibitor crizotinib. We show here that 3D spheroids of ST3GAL4 or ST3GAL6 overexpressing MKN45 gastric cancer cells are less affected by the inhibitor. In addition, we disclose a potential compensatory pathway via activation of the Insulin Receptor upon crizotinib treatment. Our results suggest that cell sialylation, in addition of being involved in tumor progression, could play a critical role in the response to tyrosine kinase inhibitors in gastric cancer.

Keywords: 3D cell culture; crizotinib; gastric cancer; glycosylation; MET; receptor tyrosine kinase; RON; sialylation; spheroids; tyrosine kinase inhibitor

1. Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer related deaths worldwide [1]. GC persists in being a clinically challenging disease due to its usual late diagnosis and high molecular heterogeneity. Despite the progress in the last decades in the cancer therapy field, there are still no effective treatments for GC nor reliable biomarkers for the prediction of therapy response or patient outcome [2].

In the scenario of personalized medicine, targeted therapies are currently the focus of cancer drug development. One strategy of the personalized therapy is the inhibition of activated signaling

pathways, either by interfering directly with receptor activation or by interrupting the downstream signaling cascade [3]. These inhibitory drugs can block the growth and spread of tumor cells by interfering with key molecules involved in malignancy, such as receptor tyrosine kinase (RTKs). The activation of RTKs is frequently altered in GC, and around 37% of patients may be potentially treatable with RTK/RAS-directed therapies [4,5]. Among these RTKs, the hepatocyte growth factor receptor (HGFR), also known as MET, has been widely studied to be involved in the oncogenesis of a large number of tumors [6]. The comprehensive molecular evaluation of gastric adenocarcinomas performed within The Cancer Genome Atlas (TCGA) project, resulted in the molecular classification of GC into four subtypes. Tumors showing marked aneuploidy and focal amplification of RTKs, including for example, exon skipping in MET, were defined as tumors with chromosomal instability [7]. The MET receptor signaling pathway is frequently overactivated in gastric cancer [6,8]. Another relevant RTK is the Recepteur d'Origine Nantais (RON) (also known as macrophage-stimulating protein receptor (MSPR) or MST1R) which commonly shows aberrant activation in various tumors due to overexpression and generation of oncogenic variants [9,10]. Alterations in the cellular glycosylation has been shown to modulate the activation of MET and RON in GC [11–13].

Glycosylation is a complex and highly regulated process of the cell that plays important roles in many cellular events, including cell-cell adhesion and communication, cell-matrix interaction, and response to microenvironmental cues. Aberrant glycosylation has a major impact on the acquisition of malignant features in the tumor progression [14–16]. The increase of sialic acid moieties in the cell surface is a shared characteristic of many tumors [17–19]. Sialic acids can be added to glycoconjugates by one of at least 20 different sialyltransferases that have different acceptor specificities [20,21].

Currently several therapeutic agents targeting MET and RON are tested in clinical and preclinical phases [9,22,23]. Crizotinib, commercialized as Xalkori®, is an oral receptor tyrosine kinase inhibitor (TKI) targeting MET, RON, and ALK [24] that was approved by the FDA for the treatment of non-small cell lung cancer in 2011. It is also currently in clinical trials for other tumors, including GC (NCT02435108, NCT03620643 and NCT02465060). Despite the great expectations placed on these targeted treatments, many patients do not respond or become resistant after treatment by mechanisms that are still not fully understood. Therefore, the assessment of the efficacy of novel treatments as well as their mechanisms of resistance must be performed using in vitro models that better simulate the in vivo conditions. In this regard, the 2D setups, which have been classically used for the culture of mammalian cells, have proven to result in limited prediction accuracy. Noteworthy, 3D cell culture systems can better mimic the complex structures and the physiological conditions of the in vivo tumor [25–28]. Multicellular tumor spheroids (MCTS), where cells form unique tridimensional complex entities, have been demonstrated to be promising for the screening of novel drugs [29–31].

In this work, we have evaluated the role of increased α 2,3-sialylation in gastric cancer therapy using an innovative 3D high-throughput cell culture methodology on a MET amplified GC cell line. Our results show that 3D spheroids of MKN45 overexpressing the sialyltransferases ST3GAL4 and ST3GAL6 are less sensitive to crizotinib treatment.

2. Results

2.1. Overexpression of α 2,3-Sialyltransferases Results in Different Cellular Glycocalyx

In a previous work, the human gastric cancer cell line MKN45, which showed low or undetectable expression levels of the sialyltransferases ST3GAL4 and ST3GAL6, respectively, was stably transfected with the expression vector containing the respective full length cDNA encoding for ST3GAL4 and ST3GAL6 [32]. We have determined the expression of the sialyltransferases ST3GAL4 and ST3GAL6 by RT-PCR after normalization with the 18S ribosomal RNA. The ST3GAL4 and ST3GAL6 clones showed an increased expression of approximately 100-fold and 10,000-fold, respectively, compared to the mock (Figure 1A). The ST3GAL6 increase could be considered a de novo expression, since its expression in both wild type and mock is negligible.

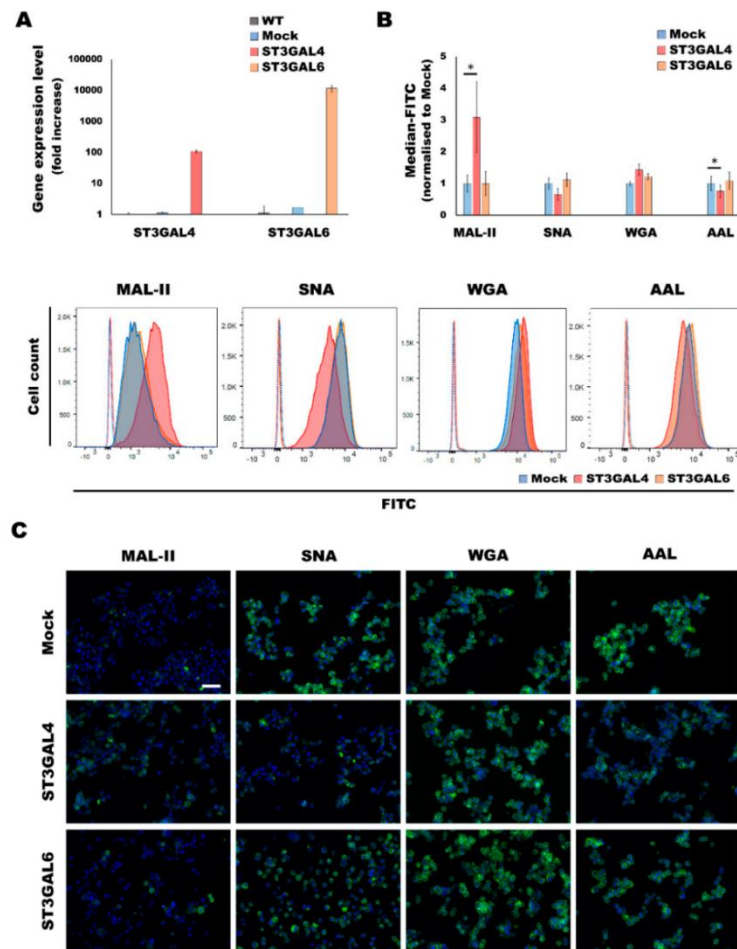


Figure 1. Characterization of the α 2,3-overexpressing cancer cell models. **(A)** Expression analysis of the ST3GAL4 and ST3GAL6 mRNA levels of the glycoengineered cell lines and the corresponding control cell lines, wild type (WT) and mock (containing the empty vector) by qRT-PCR. Data represents the average $\pm$ SD of 3 technical replicates. **(B)** Flow cytometry analysis of a panel of lectins in MKN45 glycoengineered cell lines as compared to the mock cell line. For each lectin, at least 2 independent experiments were performed. A representative plot for each lectin is also depicted. The negative controls are shown in dotted lines. Statistical significance was determined by student's *t*-test (*p*-value < 0.05 was considered significant, indicated as *). **(C)** Lectin fluorescent staining of a panel of lectins for sialylation and fucosylation characterization of the gastric cancer cell lines. The cytometry graphs and the immunofluorescence images are representative results of at least 2 independent replicates. Scale bar represents 50 μ m.

The overexpression of the α 2,3-sialyltransferases ST3GAL4 and ST3GAL6 led to the expression of a different glycosylation profile in the cell line (Figure 1B,C). The panel of lectins used to characterize the glycocalyx of cells that overexpress ST3GAL4 or ST3GAL6 consisted of Maackia amurensis lectin II (MAL-II), which recognizes sialic acid with α 2,3-linkage and Sambucus nigra agglutinin (SNA) which preferentially binds to α 2,6-sialic acid moieties. Additionally, we have also addressed the reactivity of the lectin Wheat germ agglutinin (WGA), which primarily binds to β 1,4-GlcNAc-linked residues but also has been reported to show binding to heavily α 2,3-sialylated glycoproteins [33].

Our results showed that the ST3GAL4 cell model displays a significant increase of α 2,3-sialylation concomitant to a decrease of α 2,6-sialylation. These results are in agreement with glycomic data previously reported [12,34]. In addition, a significant decrease in fucosylation, presumably outer arm fucosylation, was found as detected by Aleuria aurantia lectin (AAL). Regarding the ST3GAL6 model, we could observe a tendency of increased α 2,3-sialylation and fucosylation (Figure 1B,C).

2.2. Overexpression of α 2,3-Sialyltransferases Affects the Response to Crizotinib

To investigate whether the different glycosylation displayed by our cell lines had an effect on the capacity of cells to respond to cancer drugs, we evaluated the GC cell models under 3D culture systems and subjected these gastric MCTS to the TKI crizotinib, a drug described to target the RTKs MET and RON. In order to analyze the viability of gastric MCTS when subjected to a range of different concentrations of the inhibitor, an automated image system was used [30] (Figure 2A). The capacity of the different glycoengineered cell line models to grow as 3D spheroids was affected by TKI treatment, and gastric MKN45 MCTS shrank with increasing concentrations of the inhibitor (Figure 2). This dose effect assay revealed a plateau effect at concentrations $\geq 0.1 \mu\text{M}$ (Figure 2B). To assess the viability of the spheroids when subjected to crizotinib, we evaluated the proliferative status of the gastric MCTS. Both the western blot and immunofluorescence indicated a markedly reduction of the proliferation at doses higher than $0.1 \mu\text{M}$ crizotinib (Supplementary Material Figure S1A). Considering the intermediate concentration of $0.01 \mu\text{M}$, in which cells were still viable, we could observe significant differences among the cell line models, with cells overexpressing ST3GAL4 or ST3GAL6 displaying higher resistance to the treatment (Figure 2B).

2.3. Analysis of the Activation of MET and RON by Overexpression of α 2,3-Sialyltransferases

A previous work with the PHA-665752 TKI suggested an increased MET activation as a potential mechanism for resistance in the ST3GAL4 overexpressing model [11]. To investigate whether this was the underlying mechanism of the increased tolerance to the TKI in our 3D model system, we performed western blot and immunofluorescence analyses of the RTK activated forms, pMET and pRON. Considering the results described in the previous section, the evaluation of the RTK activation was performed using the intermediate inhibitory concentrations of 0.05 and $0.1 \mu\text{M}$ of crizotinib as well as the complete inhibition ($1 \mu\text{M}$).

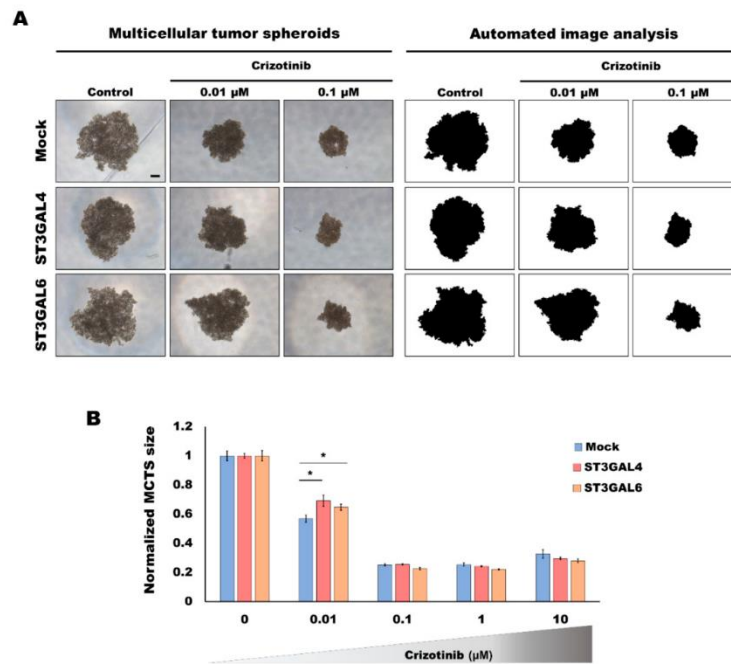


Figure 2. Analysis of the response to tyrosine kinase inhibitor (TKI) treatment of the α 2,3-overexpressing cancer cell models grown in 3D. Response to crizotinib treatment evaluated by automated image analysis of gastric multicellular tumor spheroids (MCTS) generated using ultra-low attachment (ULA) 96-well round-bottomed plates for 5 days and treated with different concentrations of crizotinib for 48 h. (A) Representative examples of the automated image analysis performed with the open-source Fiji software to determine the size of the gastric MCTS from images acquired with a Leica DMi1 microscope. Scale bar represents 200 μ m. (B) Graph represents the size variation in each spheroid after 48 h of treatment. Values are means $\pm$ SD of at least $n = 3$ spheroids. For each condition, 2 independent experiments were performed. Statistical significance was determined by student's *t*-test (*p*-value < 0.05 was considered significant, indicated as *).

The analysis of RTK activation by western blot did not show an increased activation in the MCTS of the glycoengineered cell models (Figure 3A). Although slight differences became evident in immunofluorescence analysis of pMET and pRON in cells overexpressing ST3GAL4 or ST3GAL6, when compared to the control cell line (Supplementary Material Figure S1B,C), the automated quantification of the fluorescent staining disclose no significant differences (Figure 3B).

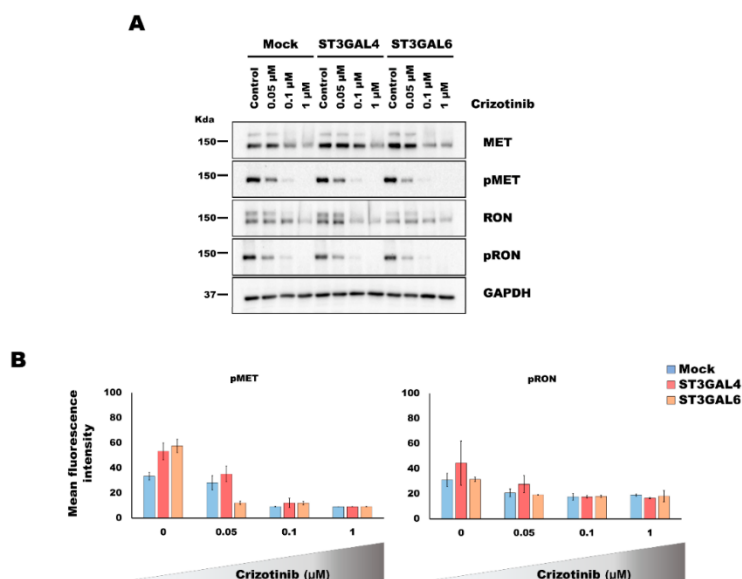


Figure 3. Evaluation of the activation of the tyrosine kinase receptors MET and Recepteur d'Origine Nantaïs (RON) in MKN45 gastric multicellular tumor spheroids (MCTS) overexpressing α 2,3-sialylation after crizotinib treatment. Gastric MCTS were generated using the 3D Petri Dish® technology (MICROTISSUES®) for 5 days and treated with different concentrations of crizotinib for 48 h. (A) Western blot analysis of MET and RON receptor tyrosine kinases and their activated forms, pMET and pRON. GAPDH was used as a loading control. (B) Results of the automated image analysis of the immunofluorescence staining of gastric MCTS (Supplementary Material Figure S1) subjected to different concentrations of crizotinib. No significant differences were found (Student's *t*-test, *p*-value > 0.05).

Concomitant to the decrease of the RTK activation, we could observe a reduction of the RTK protein expression, especially of the immature form (corresponding to the higher molecular weight band). We cannot exclude that the treatment and the consequent decreased activation of cellular signaling could alter gene expression and stability of RTK expression levels.

The results obtained with crizotinib were further validated with a different MET inhibitor, PHA-665752, displaying the same tendencies (Supplementary Material Figure S2).

2.4. Overexpression of α 2,3-Sialyltransferases Promotes Survival through the Activation of Insulin Receptors

Cancer cells are complex entities that can modulate their activity under stress conditions and thereby bypass the targets of inhibitory molecules. To monitor the changes in RTK activation upon TKI treatment, we evaluated these targets applying a phospho-RTK array. The results showed that most RTKs were less activated upon treatment. This is in accordance with the crosstalk between the members of the RTK superfamily [35]. However, in cells overexpressing ST3GAL4, the Insulin receptor (IR) showed a 1.7-fold increase after treatment (whereas mock 0.7-fold change) (Figure 4). Remarkably, the 3 members of the insulin family (IR, IGF-IR, and ALK) showed to be increasingly activated in the ST3GAL4 model, when compared to the mock treated with crizotinib, suggesting the activation of insulin family RTKs as a compensatory mechanism, not observed in the control cell line.

Thomsen-Friedenreich (TF) and the MSI status of GC tumors has been described, highlighting this glycan antigen as a biomarker with potential consequences for patient stratification [40].

Previous work disclosed that MET and RON downstream signaling is more activated upon overexpression of ST3GAL4 [11,12]. The expression of the SLeX epitope was detected by antibody-based assays, however, a detailed glycomic analysis by MS made clear that the most striking alteration in ST3GAL4 model was the general increase of α 2,3-sialylation [34]. Increased sialylation on RTKs have been shown to play a role in tumor progression [11,41]. Moreover, this altered glycosylation has been proven to lead to different response to monoclonal antibodies or TKIs. It has been recently shown that increased cell sialylation induced lower response of GC cells to the anti-ErbB2 monoclonal antibody trastuzumab [42] as well as reduced response of ovarian cancer cells to the EGFR TKI gefitinib [43]. Considering the high molecular heterogeneity of GC, we can speculate that a minor proportion of cancer cells displaying a differential sialylation could bypass the initial therapy and proliferate, giving rise to a non-sensitive tumor and ultimately leading to clinical resistance, collectively, reinforcing sialylation as a mechanism of cancer therapy resistance. In agreement with this, the analysis of the sialylation status of a resistant and a sensitive to gefitinib lung cancer cell line resulted in an overall sialylation increase of the resistant cells [44].

Crizotinib, a TKI for which we have shown promising in vitro results for the treatment of GC presenting MET amplification/overactivation, is already in clinical setting for the treatment of EML4-ALK-translocated (ALK-positive) non-small cell lung cancer (NSCLC) patients. In GC, 5% of patients present overactivation of the MET receptor and therefore, could benefit from crizotinib treatment, as also shown by others [45,46]. Our results show that increased α 2,3-sialylation leads to reduced sensitivity to the TKI, including increased resistance to ALK inhibition. The slight differences observed by the two different models could be attributed to the fact that ST3GAL6 is known to be involved in sialylation of glycolipids rather than glycoproteins [47]. Glycolipids, such as gangliosides, have been shown to be capable of altering RTK activation [14,48]. Nevertheless, both glycolipids and glycoproteins are main components of the cell glycocalyx [15] and, thus, the overexpression of the sialyltransferases ST3GAL4 and ST3GAL6 may play a role in the remodeling of the extracellular matrix of gastric MCTS. It is known that the activation of RTKs can be modulated via interaction with sialylated glycosphingolipids or other glycoconjugates, such as glycosaminoglycans or proteoglycans, present in the extracellular milieu [49–51]. Moreover, growth factors, including Hepatocyte growth factor (HGF, the ligand for MET activation), are tethered to the cell surface by interaction with glycoconjugates, facilitating ligand-mediated receptor activation and downstream signal transduction [52,53].

One of the classical mechanism to acquire resistance to RTK inhibition is the activation of an alternative RTK that restores the signaling pathways [38]. In the present work, we have performed an evaluation of the phospho-RTK proteome and disclosed the activation of the IR in the ST3GAL4 overexpressing cells in response to the inhibition of the MET and RON RTKs using an innovative 3D-approach. Moreover, previous glycoproteomic analysis showed that IR displayed increased sialylation on N-glycan site N445 in the ST3GAL4 overexpressing cell model in comparison to the mock cells [34]. We cannot clearly implicate a direct crosstalk between MET and IR, however, we hypothesize that other intermediaries or indirect crosstalk may be important as has been shown with intracellular Src kinase [54]. On the other hand, MET has been implicated as a mediator of resistance to therapies targeting members of the ErbB-RTK family in breast, lung, colon, and GC cells [55–59]. Considering that several studies reveal a particular weakness in strategies targeting single receptors, the future of the targeted therapy field points towards a “rationally-designed cocktail” of targeted drugs and the development of inhibitors targeting multiple RTK or common downstream signaling proteins [60,61]. Moreover, combination therapy could be used to tackle both acquired and intrinsic resistance. Furthermore, the use of molecules targeting N-glycosylation, such as the small molecule NGI-1, have shown promising results as a second-hit for cancer cells addicted to RTKs activation and resistant to TKIs [62]. Further development of 3D-cell culture techniques, especially the automation

and additional advances of the high-throughput technologies, will certainly contribute to the progress in the drug discovery field.

Overall, our results highlight the relevance of sialylation in cancer therapy response variability. Importantly, it is the first time to the best of our knowledge that the role of glycosylation in GC therapy response has been addressed using 3D-culture. Our system mimics better the tumor features, resulting in an improved model for in vitro drug screening. Moreover, these results lay the basis for future studies using cell glycosylation as a biomarker for prediction of therapeutic response in cancer.

4. Materials and Methods

4.1. Cell Culture

The gastric carcinoma cell line MKN45 was obtained from the Japanese Cancer Research Bank (Tsukuba, Japan) and was stably transfected with the full length human ST3GAL4 and ST3GAL6 genes and the corresponding empty vector pcDNA3.1 (mock) as previously shown [32]. The cells were grown in monolayer culture in uncoated cell culture flasks. Cells were maintained at 37 °C in an atmosphere of 5% CO₂, in RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Biowest, Riverside, MO, USA) and in the presence of 0.5 mg/mL G418 (Invitrogen, Waltham, MA, USA). Cultured cell lines were routinely tested for mycoplasma contamination by PCR amplification for mycoplasma pulmonis UABCTIP, mycoplasma penetrans HF-2, and mycoplasma synoviae 53.

4.2. RNA Extraction, cDNA Synthesis, and Real-Time Reverse Transcription PCR (RT-PCR)

Total cellular RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration and purity were determined by NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). The reverse transcription was performed with 3 µg of RNA, using random primers and the SuperScript[®] IV Reverse Transcriptase Kit (Invitrogen) following the manufacturer's instructions. RT-PCR was performed with 2 µL of diluted cDNA, 10 mM of each primer, 5 µL SYBR[®] Green Master Mix (1X) (Thermo Fischer Scientific; former Savant, MA, USA) and ultrapure water in a final volume of 10 µL using the ABI 7500 (Applied Biosystems, Foster City, CA, USA). Negative controls without templates were added for each reaction. Human 18S ribosomal RNA (18S rRNA) was used as housekeeping gene to normalized the data. Thermal cycling conditions used were an initial denaturing step at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curve analyses were performed to ensure the specificities of the amplification reactions. Relative expression values and standard deviation (SD) have been calculated using the $\Delta\Delta C_T$ approach, as previously described [63].

The primer sequences used were as follows: forward 5'-cctgtagctttcaaggcaatg-3', reverse 5'-cttttcgcaccgccttct-3' (ST3GAL4), forward 5'-cggctgattttagaagattgctt-3', reverse 5'-cggctgattttagaagattgctt-3' (ST3GAL6), and forward 5'-cgccgctagagtgaaattc-3', reverse 5'-cattcttgcaaatgcttgc-3' (18S rRNA).

4.3. Generation of Gastric Multicellular Tumor Spheroids

Gastric multicellular tumor spheroids (MCTS) formation was conducted by two different approaches, either in ULA plates (Corning, New York, NY, USA) or in 3D Petri Dish[®] (MICROTISSUES[®] technology, MicroTissues Inc., Sigma-Aldrich, St. Louis, MO, USA) following the protocol described in [30,64]. Briefly, 12-series agarose micro-molds were prepared using 2% agarose in 0.9% of NaCl. Micro-molds were placed in 12-well plates and equilibrated with RPMI 10%FBS before cell seeding. Cell suspensions of 250 cells/microwell of each cell line were seeded in the corresponding micro-mold. A 30 min incubation period was used for cells to settle into the micro-molds and additional medium was finally added to the wells. Spheroids formation both in ULA plates and 3D Petri Dish[®] was

monitored with the inverted microscope for cell culture Leica DMI1 (Leica Microsystems, Wetzlar, Germany).

4.4. Spheroid Treatment

The MCTS response to the RTK inhibitors crizotinib and PHA-665257 (both from Sigma-Aldrich) was followed by monitoring the spheroids growth. Aliquots of 250 cells/well were seeded in the ULA plates. After 5 days, when the gastric MCTS were formed, cells were treated with the inhibitors diluted in cell culture medium at concentrations ranging from 0.01 to 10 μ M for 48 h. Control conditions containing the corresponding amount of the vehicle (DMSO) were performed. At day 5 and 7 (before and after treatment), microscope images were acquired using a Leica DMI1 microscope.

For immunofluorescence and protein analyses (western blot and array), after 5 days of MCTS formation in molds as described in Section 4.3, cells were treated with the corresponding TKI concentration (as well as the vehicle control) for 48 h.

4.5. Image Analysis of Size of the Gastric Multicellular Tumor Spheroids

The gastric MCTS size was examined as previously described in [30] using the ImageJ image analysis software with the Fiji image processing package [65]. Briefly, a picture of each well of the ULA plate containing one spheroid each was taken at the different time points. All images were processed by color deconvolution “H and E” using the green channel, threshold “minimum” was applied and only objects larger than 62,000 pixels with “include holes” were kept. The resulting mask, which contained a single object, was used to define spheroid size. The size difference for each spheroid was analyzed and the results subjected to statistical analysis. Each independent experiment consisted of a ULA plate containing all the tested conditions in at least triplicates. In order to calculate the Normalized MCTS size, we applied the following calculations:

1. Ratio of MCTS for each MCTS (x):

$$\text{Ratio MCTS (x)} = \frac{\text{Area MCTS (x) after 48h of treatment}}{\text{Area MCTS (x) before treatment}}$$

2. Normalized ratio for each MCTS (x):

$$\text{RaNormalized Ratio MCTS(x)} = \frac{\text{Ratio MCTS (x)}}{\text{Average Ratio of control condition (0}\mu\text{M)}}$$

4.6. Flow Cytometry and Lectin Fluorescent Staining

Cells were detached using Gibco® versene solution (ThermoFisher, Waltham, MA) and stained with previously conjugated lectins with FITC-conjugated streptavidin (Invitrogen) for 20 min at 4 °C. Cells were strained, labeled with propidium iodide and measured using BD FACSCanto™ II (BD Biosciences, San Jose, CA, USA) Three independent experiments were conducted. Data were analyzed using FlowJo (BD Biosciences, San Jose, CA, USA). 4.4. The biotinylated lectins used were MAL-II (*Maackia amurensis* lectin II), SNA (*Sambucus nigra* agglutinin), WGA (Wheat germ agglutinin) and AAL (*Aleuria aurantia* lectin), (all from Vector Laboratories, Burlingame, CA, USA).

An aliquot of each sample was placed on microscopy slides and let dry overnight at RT in dark. Nuclei were stained with DAPI and slides were mounted with VectaShield (Vector Laboratories) and imaged with Zeiss Axio Imager Z1 microscope equipped with an AxioCam MR ver.3.0 (Carl Zeiss, Oberkochen, Germany).

4.7. Fluorescent Cell Staining of Gastric Spheroids

The gastric MCTS generated with 3D Petri Dish® (as described in Section 4.3) and treated (as described in Section 4.4) with different amounts of crizotinib and PHA-665257 were fixed with 4% *w/v* PFA for 30 min at RT. Fixed samples were paraffin-embedded and the blocks were cut into 5 µm sections. Slides were deparaffinized in xylene and rehydrated in sequentially decreasing ethanol concentrations prior to immunofluorescence staining. In brief, slides were blocked with non-immune goat serum in 10% PBS and incubated overnight with the corresponding dilution of the primary anti-human antibody. Slides were then washed with PBS and incubated with the secondary antibody anti-rabbit IgG conjugated with Alexa Fluor®-488 for 1 h at RT. Nuclei were counterstained with DAPI and samples were mounted with VectaShield (Vector Laboratories). Sections were imaged with Zeiss Axio Imager Z1 microscope equipped with an AxioCam MR ver.3.0 (Carl Zeiss).

The antibodies used were: p-MET (Tyr1234/1235, D26 clone, Cell Signaling Danvers, MA, USA), and p-RON (Y1238/Y1239, R&D Systems, McKinley Place, MN, USA). These antibodies were used after antigen retrieval with Tris/EDTA pH 9 solution (Novocastra™ Epitope Retrieval Solutions, Leica Biosystems) and diluted 1/100. Ki-67 (ab15580, Abcam, Cambridge, UK, dilution 1/500) used after antigen retrieval with citrate buffer pH 6.

4.8. Image Analysis of Receptor Activation

The fluorescent staining from MET and RON activation upon crizotinib treatment of the gastric MCTS described in Section 4.7 was examined using the ImageJ image analysis software with the Fiji image processing package [65]. All images were analyzed by applying the “color threshold” function to subdivide the pixels of the green channel into groups based on their intensity. The pixels of each group were quantified and the result was used to calculate a mean intensity value for each image. At least 2 different images per condition were analyzed.

4.9. Western Blot

Total protein lysates were collected from 48-h-treated gastric MCTS (described in Section 4.4) using lysis buffer 17 (R&D Systems, Minneapolis, MN, USA) supplemented with 1 mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO, USA), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) and cOmplete™ protease inhibitor cocktail (Roche, Basel, Switzerland; Sigma-Aldrich). Protein concentration of each condition was determined using the DC protein assay (BioRad, Hercules, CA, USA). Same amounts of total protein lysates were electrophoresed under reducing conditions on polyacrylamide gels and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Chicago, IL, USA). After 1 h blocking at room temperature, membranes were incubated overnight at 4 °C with primary antibodies. Then, membranes were washed and incubated for 1 h at room temperature with the corresponding peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Cambridgeshire, UK). Chemiluminescence signal was obtained using the ECL western blot detection reagent GE Healthcare and visualised with Chemidoc (BioRad).

The antibodies used were: MET (3D4, ThermoFisher Scientific, Waltham, MA, USA, dilution 1/1500), RON (E-3, Santa Cruz Biotechnology, Dallas, TX, USA, dilution 1/100), p-MET (Tyr1234/1235, D26 clone, Cell Signaling Danvers, MA, USA, dilution 1/1500), p-RON (Y1238/Y1239, R&D Systems, McKinley Place, MN, USA, dilution 1/500), GAPDH (0411, Santa Cruz Biotechnology, dilution 1/2000), and Ki-67 (ab15580, abcam, Cambridge, United Kingdom, dilution 1/500).

4.10. Phosphoprotein Array

Five-day gastric MCTS were treated with 0.06 µM of crizotinib for 48 h (as described in Section 4.4) and lysed in lysis buffer 17 (R&D Systems) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration of the lysates was determined by the DC protein assay (BioRad) and the

recommended total protein amounts were used for the human phospho-RTK (R&D Systems). The array protocol and the subsequent analysis of the results were performed according to manufacturer's instructions. The optical density signal of each spot was quantified using the Image Lab Software (BioRad) under non-saturating conditions. Upper-left corner spots were not quantified due to saturation and overlapping signal. Each RTK is present in duplicate in the array, therefore each bar in the graph represents the average of the signal of the two spots of the treated sample normalized to the control (non-treated). A value equal to 1 means no difference between crizotinib-treated and untreated sample. A value higher or lower than 1 represents either increased activation or inactivation of the RTK upon treatment, respectively.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/21/3/722/s1>.

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Abbreviations

AAL	<i>Aleuria aurantia</i> lectin
EGFR	Epidermal growth factor receptor 1
ErbB2	Epidermal growth factor receptor 2
GC	Gastric cancer
HGFR	Hepatocyte growth factor receptor
IR	Insulin receptor
MAL-II	<i>Maackia amurensis</i> lectin II
MCTS	Multicellular tumor spheroids
MSI	Microsatellite instable
NSCLC	Non-small cell lung cancer
RON	Recepteur d'Origine Nantais (also known as macrophage-stimulating protein receptor (MSPR) or MSTIR)
RT-qPCR	Real-time quantitative PCR
RTK	Receptor tyrosine kinase
SNA	<i>Sambucus nigra</i> agglutinin
TF	Thomsen-Friedenreich
TKI	Tyrosine kinase inhibitor
ULA	Ultra-low attachment
WGA	<i>Wheat germ</i> agglutinin

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SUPPLEMENTARY FILES

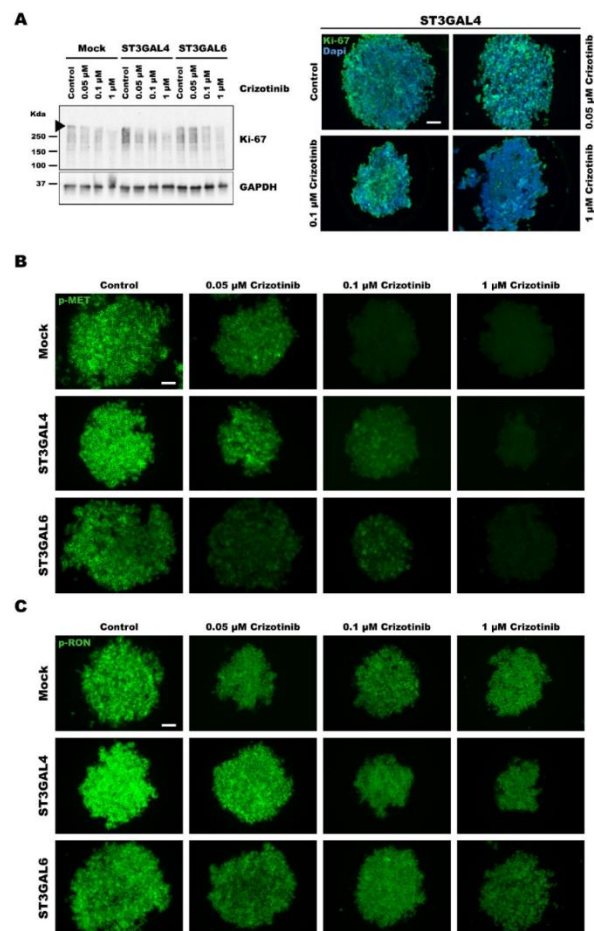


Figure 1. Analysis of the response to TKI treatment crizotinib of the $\alpha 2,3$ -overexpressing cancer cell models grown in 3D. Gastric MCTS were generated using the 3D Petri Dish® technology (MICROTISSUES®) for 5 days and treated with different concentrations of PHA-665257 for 48 h. (A) Western blot and immunofluorescent staining of the proliferation marker Ki-67 of gastric MCTS (B-C). Immunofluorescent staining of (B) pMET and (C) pRON of the treated gastric MCTS. Scale bar represents 50 μ m.

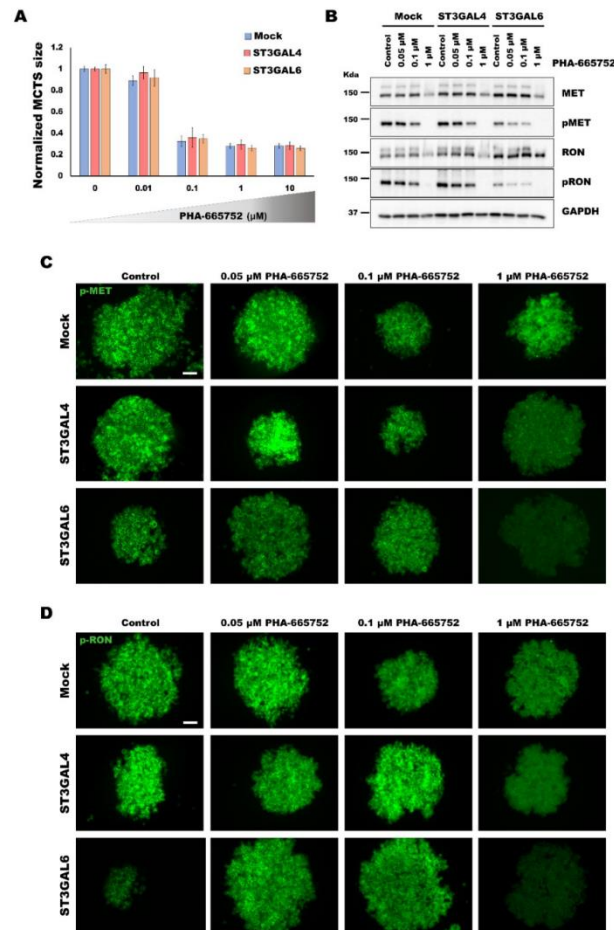


Figure S2. Analysis of the response to TKI PHA-665752 treatment of the α 2,3-overexpressing cancer cell models grown in 3D. (A) Gastric multicellular tumor spheroids (MCTS) were generated using ultra-low attachment (ULA) 96-well round-bottomed plates for 5 days and treated with different concentrations of PHA-665257 for 48 h. Response to PHA-665752 treatment evaluated by size using automated image analysis. Graph represents the size variation in each spheroid after 48 h of treatment. Values are means $\pm$ SD of at least n=3 spheroids. For each condition, 2 independent experiments were performed. (B-D) Gastric MCTS were generated using the 3D Petri Dish® technology (MICROTISSUES®) for 5 days and treated with different concentrations of PHA-665257 for 48 h. (B) Western blot analysis of MET and RON receptor tyrosine kinase and their activated forms, pMET and pRON. GAPDH was used as a loading control. (C) Immunofluorescent staining of pMET and (D) pRON of the treated gastric MCTS. Scale bar represents 50 μ m.

APPENDIX III

Scientific Outputs

SCIENTIFIC OUTPUTS

Journal Articles:

- Meritxell Balmaña, **Francisca Diniz**, Tália Feijão, Cristina C. Barrias, Stefan Mereiter, Celso A. Reis (2020). Analysis of the Effect of Increased α 2,3-Sialylation on RTK Activation in MKN45 Gastric Cancer Spheroids Treated with Crizotinib. *Int. J. Mol. Sci.*, 21(3), 722
- Beatriz Leão, Xiaogang Wen, Henrique Duarte, Irene Gullo, Gilza Gonçalves, Patrícia Pontes, Claudia Castelli, **Francisca Diniz**, Stefan Mereiter, Joana Gomes, Fátima Carneiro, Celso Reis (2021). “Expression of Thomsen-Friedenreich Antigen in colorectal cancer and association with microsatellite instability”. *Int. J. Mol. Sci.*
- **Francisca Diniz**, Pedro Coelho, Henrique O. Duarte, Bruno Sarmento, Celso A. Reis, & Joana Gomes (2022). Glycans as Targets for Drug Delivery in Cancer. *Cancers*, 14(4), 911.
- **Francisca Diniz**, Maria Azevedo, Flávia Sousa, Hugo Osório, Diana Campos, Paula Sampaio, Joana Gomes, Bruno Sarmento & Celso A. Reis (2022). Polymeric nanoparticles targeting Sialyl-Tn in gastric cancer: A live tracking under flow conditions. *Materials Today Bio*, 100417.
- **Francisca Diniz**, Sofia Lamas, Hugo Osório, Paulo Aguiar, Daniela Freitas, Fátima Gartner, Bruno Sarmento, Celso A. Reis, Joana Gomes (2022). Nanoparticles targeting Sialyl-Tn for efficient tyrosine kinase inhibitor delivery in gastric cancer. (Manuscript Under Revision)

Patents:

- Co-Inventor of the Patent, number 20221000002083 (17-05-2022). Provisional patent application in the National Institute of Industrial Property, Lisbon, Portugal. Inventor: Celso A. Reis.

Oral communications (Speed talks):

- **Francisca Diniz**, Maria Azevedo, Flávia Sousa, Hugo Osório, Paula Sampaio, Joana Gomes, Bruno Sarmento, Celso A. Reis. “Innovative Nanodelivery system using glycans for targeted delivery in Cancer.” 4th PhDay: Break Boards and Build Opportunities, 30 November 2021, i3S, Porto, Portugal.

• **Francisca Diniz**, Maria Azevedo, Flávia Sousa, Hugo Osório, Paula Sampaio, Joana Gomes, Bruno Sarmento, Celso A. Reis. “Polymeric Nanoparticles Targeting Glycans in Gastric Cancer Cells Under Live Flow Conditions”. 32nd Annual Conference of the European Society for Biomaterials 2022, 4-8 September 2022, at Bordeaux, France.

Posters communications:

• **Francisca Diniz**, Meritxell Balmaña, Stefan Mereiter, Tália Feijão, Cristina C. Barrias, Celso A. Reis. “Multicellular Human Gastric Cancer Spheroids Mimic the Glycosylation Phenotype of Gastric Carcinomas”. Interrogations at the Biointerface - 7th Advanced Summer School, 27-28 June 2019, Porto, Portugal.

• **Francisca Diniz**, Meritxell Balmaña, Stefan Mereiter, Tália Feijão, Cristina C. Barrias, Celso A. Reis. “Multicellular Human Gastric Cancer Spheroids Mimic the Glycosylation Phenotype of Gastric Carcinomas. GLUPOR - 13th International Meeting of the Portuguese Carbohydrate Group, 3-5 September 2019, Porto, Portugal.

• **Francisca Diniz**, Meritxell Balmaña, Stefan Mereiter, Tália Feijão, Cristina C. Barrias, Celso A. Reis. “Human Gastric Cancer Spheroids: evaluation of glycosylation and implications of tyrosine kinase inhibitors treatment.” Goodbye Flat Biology: Advancing 3D-based Models for Cancer Biology and Drug Discovery, 10 - 13 November 2019, Berlin, Germany.

• **Francisca Diniz**, Flávia Sousa, Diana Campos, Catarina Gomes, Joana Gomes, Bruno Sarmento, Celso A. Reis. “Sistema inovador de nanoentrega usando glicanos para entrega direcionada em cancro”. 2º Encontro Nacional de Jovens Investigadores em Oncologia, 24 September 2020, Porto, Portugal.

• **Francisca Diniz**, Maria Azevedo, Flávia Sousa, Hugo Osório, Paula Sampaio, Joana Gomes, Bruno Sarmento, Celso A. Reis. “Polymeric Nanoparticles Targeting Glycans in Gastric Cancer Cells Under Live Flow Conditions”. 32nd Annual Conference of the European Society for Biomaterials 2022, 4-8 September 2022, at Bordeaux, France.

Awards:

• Best Poster Communication: 32nd Annual Conference of the European Society for Biomaterials 2022, 4-8 September 2022, at Bordeaux, France.

• Training School “Conjugation of glycans onto nanomaterials: opportunities in therapy and diagnosis”, 12-14 of September 2022, at Strasbourg, France.

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