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Modulatory Effects of Deacetylated Sialic Acids (a Sugar Residue) in NK-Mediated Cytotoxicity and Targeted Therapy by Receptor Tyrosine Kinase Inhibitors

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MODULATORY EFFECTS OF DEACETYLATED SIALIC ACIDS (A SUGAR RESIDUE) IN NK-MEDIATED CYTOTOXICITY AND TARGETED THERAPY BY RECEPTOR TYROSINE KINASE INHIBITORS

BY

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DISSERTATION ACCEPTANCE PAGE
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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Nicole Lounsbery, PhD
Director, Graduate School Date

I dedicate this dissertation to God Almighty who made it possible through His unending love, grace and protection. Secondly, I dedicate this to my parents, Mr. Franklin Kwao Anim and Mrs. Lucy Aba Anim.

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CONTENTS

ABBREVIATIONS

APC	Allophycocyanin
DMEM	Dulbecco's Modified Eagle Medium
EGFR	Epidermal Growth Factor Receptor
G_1	Gap 1 phase (cell cycle)
G_2	Gap 2 phase (cell cycle)
IC_{50}	Concentration needed to obtain 50% inhibition
mAb	Monoclonal antibodies
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RPMI	Rosewell Park Memorial Institute Medium
SIGLECS	Sialic-acid-binding immunoglobulin-like lectins

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ABSTRACT

Modulatory Effects of Deacetylated Sialic Acids (a Sugar Residue) in NK-Mediated Cytotoxicity and Targeted Therapy by Receptor Tyrosine Kinase Inhibitors

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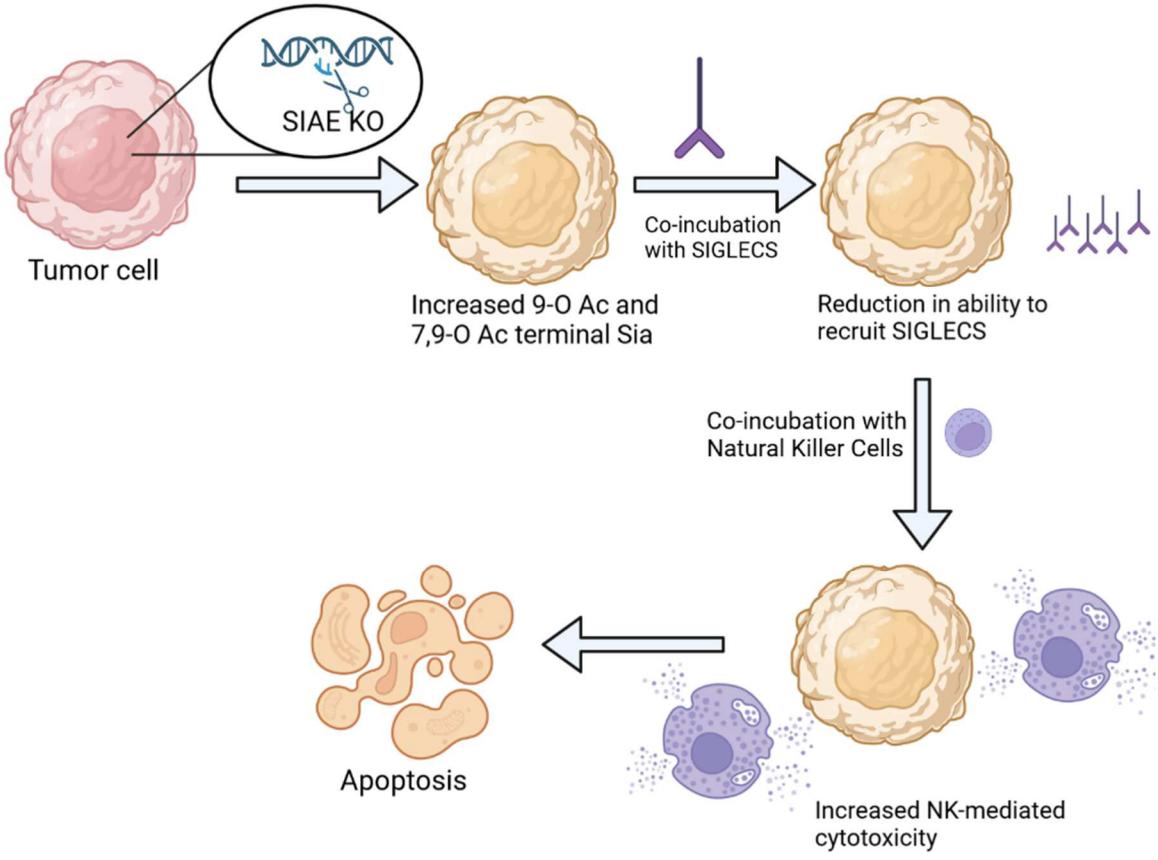
The complex nature of the biology of cancer is still an unraveling science, yielding several biomarkers that have served as molecular targets for detection and treatment of the disease. How sugars, glycans, play a role has remained relatively uninvestigated. Sialic acid (Sia), a sugar residue on the surface of cells, has been identified as a hallmark of cancer and its progression. Sialic acid can be highly functionalized, but we became interested in acetylated Sias. This functional group is modulated by Sialate O- acetylesterase (encoded by the gene SIAE) and Sialate O-acetyltransferase (encoded by CASD1), enzymes that play a crucial role in functionalizing sialic acid. CASD1 and SIAE catalyze the addition and removal of acetyl groups to sialic acids respectively. We showed that modulating SIAE and CASD1 interferes with ligand-receptor binding interactions with adjacent cell surfaces, specifically, we demonstrated that deacetylated Sias reduce NK-mediated cytotoxicity of colon and lung cancer cells via Siglec binding.

Building upon this study we turned our attention to understanding how these genes modulate the cell's susceptibility to drugs. Our findings show that targeting SIAE and

CASD1 in chemotherapy renders it more susceptible to drugs that target and inhibit tumor cell proliferation via EGFR signaling. The tested novel hybrid analogs (MMA 294, 320 and 321) and Sorafenib exhibited a dose dependent cytotoxicity via apoptosis on colon and lung cancer cells. The effect of these drugs on the cell cycle of HCT116 and A549 cells was also assessed using DNA staining with propidium iodide. MMA 321 and sorafenib elicited a G_0/G_1 cell cycle arrest in HCT116 and A549 WT cells but a G_2/M in HCT116 and A549 SIAE. Further screening of phosphorylated EGFR by immunofluorescence revealed an overexpression of pEGFR in the CASD1 and SIAE knockout cells suggesting an increased activation of this growth receptor rendering these CRISPR Cas-9 knockout cells highly proliferative but extremely sensitive to the novel hybrid analogs of estrone origin.

Chapter 1

Deacetylated Sialic Acids Modulates Immune Mediated Cytotoxicity Via The Sialic Acid-Siglec Pathway



1.1 Abstract

Cancers utilize glycans to evade the immune system via the Sialic acid (Sia)-Siglec (Sialic-acid- binding immunoglobulin-like lectins) pathway. Specifically, atypical structural forms of sialic acid bind to inhibitory Siglec receptors on natural killer (NK) cells resulting in the suppression of immune cell mediated cytotoxicity. The mechanism of action that governs the Sia-Siglec pathway in cancers is not understood. Specifically, how deviations from the typical form of Sia mechanistically contribute. Here, we focused on modulating 9-O and 7, 9-O-acetylation of Neu5Ac, via CRISPR- Cas9 gene editing, a functional group that is absent from Sias on many types of cancer cells. The two genes that are responsible for regulating the level of acetylation on Neu5Ac, are Sialic acid acetylesterase (SIAE) and Sialic acid acetyltransferase (CASD1). These genes modulated Siglec binding in colon, lung and a noncancerous kidney cell line. In the absence of SIAE, Neu5Ac is acetylated, engagement of cancer associated Siglecs is reduced while binding was increased when the ability to acetylate was removed via CASD1 knock out. In the absence of SIAE NK mediated cytotoxicity increased in both colon and lung cancer cells. In addition to modulating Siglec binding, SIAE expression modulates the level of Sias in a cell, and the α2–6-linkage of Sias—which is specifically upregulated and associated with cancers. Uncovering how functional group alterations on Neu5Ac contribute mechanistically to both Siglec receptor binding, the Sia-Siglec immune evasion pathway, and the production of cancer associated glycosidic linkages—offers a promising avenue for targeted cancer immune therapies in the future.

1.2 Introduction

1.2.1 Cancer Is A Menace To Public Health

Cancer is one of the leading causes of deaths in developed countries, comparable only to cardiovascular diseases (Bray *et al*., 2021; Sung *et al*., 2021). Across the globe, it is estimated to have caused 9,958,133 deaths during the COVID pandemic in 2020 (Sung *et al*., 2021). Comparing the US to China, the disease burden of cancer has been seen to decrease gradually in the US while increasing steadily in China. The United States has been reported to have gradually adopted better behaviors associated with cancer risks and medical practices such as improved cancer screening tests, better treatment regimens as of a result of successful progress in cancer research, its prevention and care (Schilsky *et al*., 2020). In 2022, it is estimated that the United States will record approximately 2,370,000 new cancer cases and 640,000 cancer deaths with lung cancer predicted to be the leading cause of cancer deaths (Xia *et al*., 2022).

Lung cancer is the leading cause of cancer deaths in the US and the world. In 2021, an estimated total number of 235,760 new lung cancer cases were reported, placing it as the second most common cancer reported (excluding non-melanoma skin cancers). Even though the age-adjusted lung cancer incidence has been reducing from 1999 to 2019 (70.8 to 52.9), it is still the number cause of cancer related deaths in the country (Zhang *et al*., 2022). Lung cancer has a very poor 5-year rate of survival of 21% (in all stages) and one

of the contributing factor to this was that over half (57%) of these lung cancer cases were diagnosed with distant metastasis rather than at localized or regional stages (Siegel *et al*., 2021).

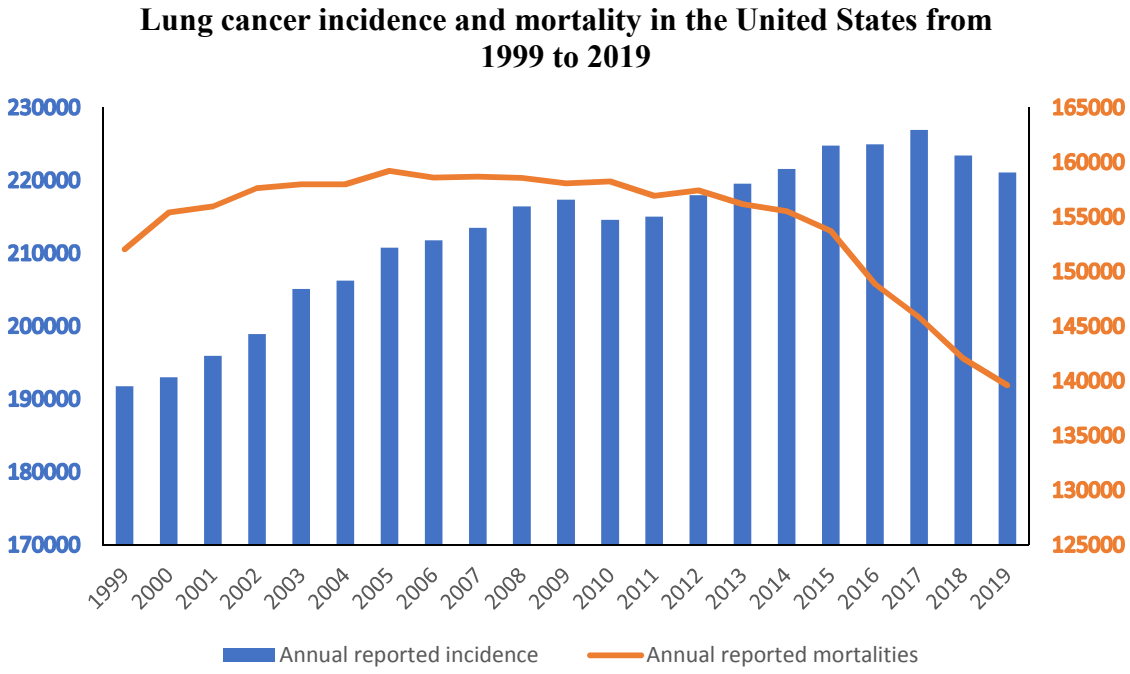


Figure 1.1 Annual rates of lung cancer cases, 1999 to 2019

On the contrary, non-small cell lung cancer (NSCLC), which accounts for 80% of all lung cancer cases has seen a rather improved 2-year rate of survival from 34% in 2009-2010 to 42% in 2015-2016 with a 5 to 6% absolute gains in all stages of diagnosis (Siegel *et al*., 2021). NSCLC has a much better rate of early detection and diagnosis than small cell lung cancers, making it possible for effective treatments regimens to be started in time before it metastasizes (Howlader *et al*., 2020). One other factor that has accounted for the decline in the overall lung cancer disease burden is the decrease in cigarette smoking in the adult population in the US. Smoking of cigarette is one of the well-known risk factors to developing lung cancer and it is reported to cause about 81.7% of lung cancers in the United

States (Islami *et al*., 2017). Since the prevalence of cigarette smoking reduced by 29.8% (in adult males) and 38.7% (in adult females) from 1990 to 19.9% (in males) and 15.3% (in females) in 2019, there is a steady decline in the rates of new lung cancer cases (Reitsma *et al*., 2021). The decrease in the prevalence of cigarette smoking is attributed to a number of very successful public health policies on smoking including increases in cigarette excise taxes, smoking restrictions in public areas, reduced access to cigarettes and increased public awareness of the dangers of smoking cigarette (Moolgavkar *et al*., 2012).

Colon cancer is considered as one of the deadliest forms of cancer, in the same league as lung, breast and prostate cancer. Colon cancer can develop in any of the four segments of the colon, namely the ascending, transverse, descending and sigmoid sections. According to the 2020 estimates from the United States, it is the third most commonly diagnosed cancer with an incidence of 40.7 per 100,000 population in the US. It was also the third leading cause of cancer related deaths, accounting for 14.8 per 100,000 population deaths in 2020 (Siegel *et al*., 2020). However, these statistics are an improvement compared relative to 20 years prior incidence and mortalities due to the presence of better and more sensitive screening methods and effective removal of precancerous lesions detected in the early onset of the disease (García-Albéniz *et al*., 2016).

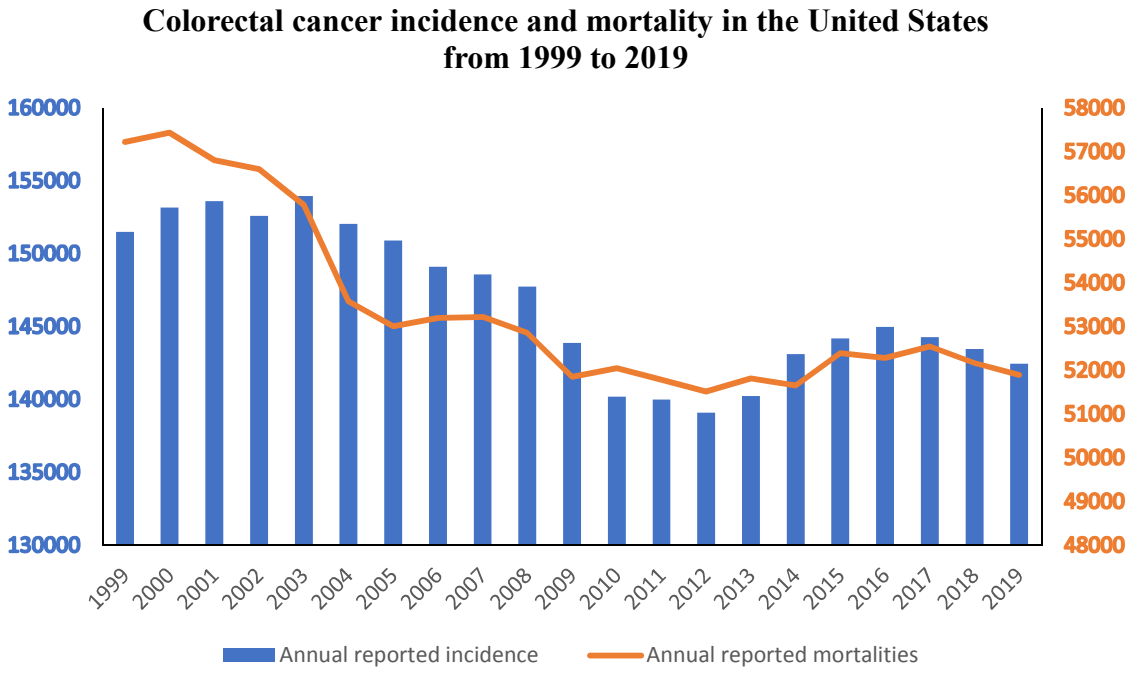


Figure 1.2 Annual rates of colorectal cancer cases, 1999 to 2019

Colon cancer has no specific cause but several risk factors that predisposes individuals to the disease. Colon cancer has been shown by meta-analysis to have some familial risk mainly between first degree relatives. About 5% of patients diagnosed with colon cancer have some form of inherited genetic predisposition (Burt and Neklason, 2005). Furthermore, certain lifestyle factors also play key roles in increasing the risk to developing colon cancer. One of such lifestyle factors is the consumption of meat diets, particularly red and processed meat which is rich in Neu5Gc, a type of sialic acid that has been linked to cancer (Orlich *et al*., 2015). Several studies have confirmed how meat and fat consumption can induce genetic and epigenetic changes in the colon that often leads to genome instability and an increased risk of mutations in tumor suppressor or oncogenes (Raskov *et al*., 2014). Such genetic changes is possible as a result of poor meat preparation, high nitrate and nitrite found in processed meats and undigested remnants of meats that

cause bacterial accumulation and fermentation and the ultimate formation of N-nitroso compounds from the action of the bacterial. N-nitroso compounds are known to readily form adducts with DNA and lead to mutations with the genome (Raskov *et al*., 2014). On the other hand, a healthier diet, high in fiber, fish, legumes (especially soybeans) and calcium has been reported by several studies to reduce the risk of developing colon cancer (Aune *et al*., 2011; Gonzalez and Riboli, 2010). Even, simple vegetarian diets can also reduce the risk of developing colon cancer (Orlich *et al*., 2015).

1.2.2 Sialic Acids Discovery

Sialic acids are a group of nine-carbon sugar residues that are found in vertebrates, eubacteria and archaea. They can also be generally referred to as nonulosonic acids and are characterized by the presence of a 3-carbon exocyclic side chain as well as one or more acylated amino groups. There are over 50 different sialic acids found in nature, with the most predominant ones being N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-nonulosonic acid (Kdn). Neu5Ac is the most common sialic acid found in humans whiles Neu5Gc and Kdn are some examples of nonhuman sialic acids (Schauer, 2004).

Sialic acids were independently identified by two different researchers, Gunnar Blix and Ernst Klenk in 1936 and 1941 respectively. Blix isolated these sugars from salivary mucins and named them sialic acids while Klenk named them neuraminic acid because he isolated them from neurons in the brain (Blix, 1936; Klenk, 1941). Sialic acids can have O-acetyl, O-lactyl, O-sulfo, O-methyl or O-phospho groups attached contributing to the vast diversity and functionality of these sugars.

1.2.3 Synthesis Of Sialic Acids

Upon entry of glucose into cells via glucose transporters, and its subsequent conversion to N-Acetylglucose-6-phosphate (GlcNAc), the bifunctional enzyme glucosamine UDP-GlcNAc-2-epimerase/N-acetylmannosamine kinase, coded by the GNE gene. This enzyme catalyzes the conversion of UDP-bound GlcNAc to N-acetylmannose-6-phosphate (ManNAc-6-P) and UDP in 2 steps. Firstly, it catalyzes the hydrolytic epimerization of UPD-GlcNAc to N-Acetylmannosamine (ManNAc), with the release of UDP from the substrate. Secondly, ManNAc is phosphorylated by the N-acetylmannosamine kinase functionality of this protein to ManNAc-6-phosphate using ATP. Nonaka et al. (1985) reported a rare disorder that affects muscles and leads to a mild serum creatine kinase elevation and muscle atrophy in some patients. This disease was later named GNE myopathy and discovered to be caused by mutations in the GNE gene and found to be inherited in an autosomal recessive manner. It has an incidence of 1-9 in 100,000 individuals and is characterized by a progressive weakness in muscles which leads to a reduced ability to grasp with the hands and a loss of balance in patients (Carrillo *et al*., 2018).

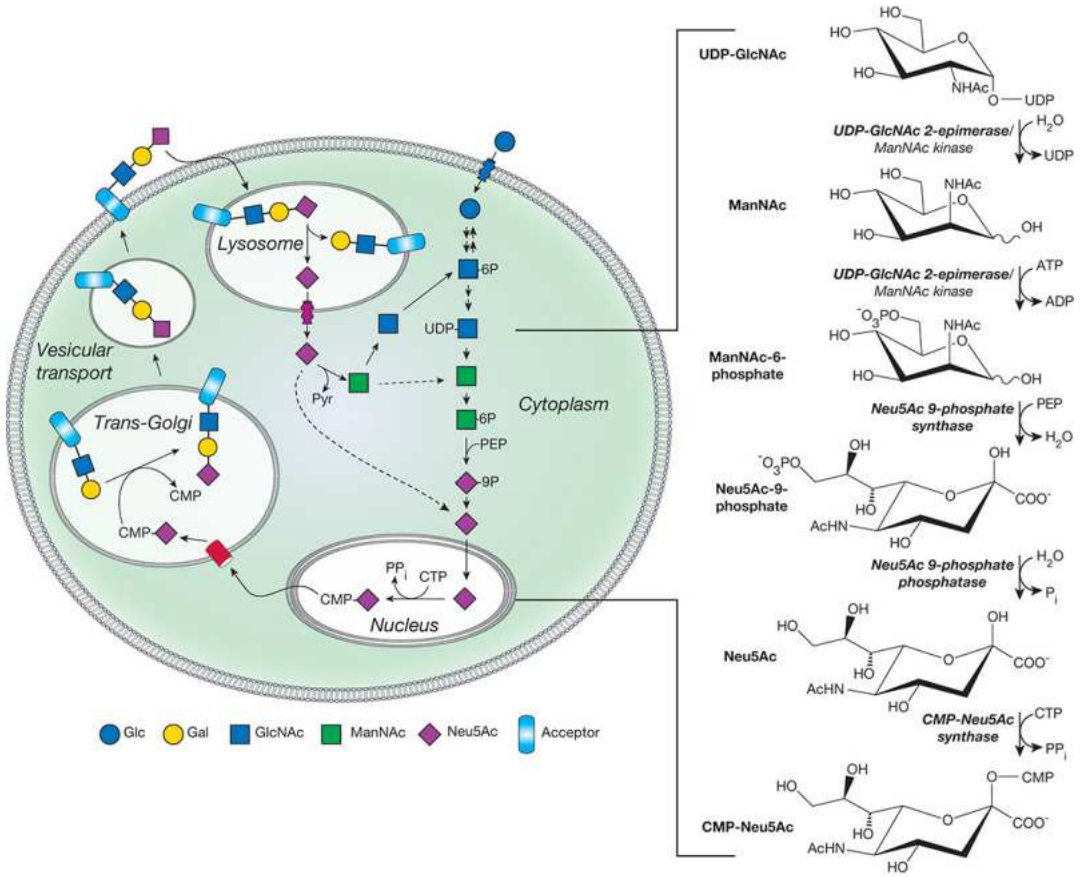


Figure 1.3 Physiological pathway highlighting the synthesis and transport of Sia

Synthesized ManNAc-6-P condenses with phosphoenolpyruvate (PEP) to produce Neu5-Ac that bears an extra phosphate group at C-9. Neu5-Ac-9-P undergoes dephosphorylation by Neu5Ac-9-phosphate phosphatase to release Neu5Ac into the cytoplasm.

1.2.4 Sialoglycans Are Synthesized By Sialyltransferases

Sialoglycans are generally carbohydrates that contain sialic acids, mainly as terminal residues. Their synthesis involves addition of sialic acids to growing glycan chains via specific linkages catalyzed by sialyltransferases (ST). Aside from the diversity in these nonulosonic acids, the specific linkages between these sugars also adds another layer of complexity to their structures, making these one of the most diversified systems within any

given population. Sialyltransferases adds Sia to the non-reducing sugar chains of proteins and lipids using cytidine monophosphate linked Sia as the donor molecule. The STs catalyze the glycan linkages between a C-2 anomeric carbon of Sia in the α configuration to the C-3 or C-6 positions of galactose, or the C-6 of N-acetylgalactosamine or the C-8 or C-9 of another Sia (Petit *et al*., 2013).

Cytosolic Sia can only be added to a glycan chain after it undergoes activation via coupling with cytosine 5'-monophosphate (CMP). This process is compartmentalized and occurs in the nucleus and catalyzed by the nuclear CMP-Neu5Ac synthase. CMP-Sia exits the nucleus and gets concentrated in the Golgi lumen, the cellular compartment that houses the sialyltransferases. The influx of CMP-Sia occurs via an antiporter that exchanges CMP for CMP-Sia, thus reducing cytosolic CMP-Sia and increasing cytosolic CMP in the process. The CMP-Sia antiporter is localized in the medial-trans Golgi and possesses two specific ER export motifs in its carboxyl-terminal cytoplasmic tail. Zhao et al. showed that the specific deletion of the last four amino acids in the C-terminal of this transporter resulted in a loss of its export signals and prevented the export of this protein from the ER (Zhao *et al*., 2006). Sialic acid levels in cells have been shown to be largely impacted by this transporter. Wong et al (2006) successfully increased glycoprotein sialylation in Chinese hamster ovary cells by overexpressing the CMP-Sia antiporter. Conversely, Chinese hamster ovary cells that possess the mutant non-functional form of this transporter lack the ability to sialylate recombinant interferon gamma, proving the therapeutic potential of this transporter as a molecular target in diseases relating to sialylation (Lim *et al*., 2008).

Humans have about 20 known genes that encode these STs and these work co-operatively to put Sia on glycans, marking the last stage of post-translational modification of proteins.

Sia have hydrophilic and electronegative features and their terminal position helps them in playing various crucial roles spanning intercellular interactions to migration and adhesion of cells to extracellular matrix. In most cancers, sialylation is poorly controlled, leading to poor prognosis of the disease. Aberrant expression of STs have been reported to be one of the mechanisms through which cancer cells enhance sialylation on their cognate glycoconjugates. This phenotype allows cancer to evade the immune system, proliferate and metastasize rapidly and resist apoptosis particularly when the Fas receptors are hypersialylated (Zhou *et al*., 2020)

1.2.5 Structural Diversity of Sialic Acids And Their Roles in Health

Sialic acids can have several structural diversities depending on the functional groups attached to the 9-carbon backbone. Such modifications can include phosphate, sulfate, methyl or acetyl groups attached to one or more hydroxyl group substituents of the molecule (Schauer, 1982). Enzymes that catalyze such reactions use molecular donors like S-adenosylmethionine (SAM) as a methyl group donor, or 3'-phosphoadenosine-5'-phosphosulphate as a sulfate group donor. This creates a vast array of diversity in the molecule and accounts for its numerous roles across several species.

Acetylation of Sia has been the most widely studied functional group and has been linked to several disease conditions. For example, O-acetylated sialic acids have been observed to be highly expressed in developing tissues of neuroectodermal origin as well as in B and T lymphocytes. In childhood acute lymphoblastic leukemia, 9-O-acetylation in

Sia can be readily used as a diagnostic or prognostic marker, helping in informing physicians of the presence and severity of the disease.

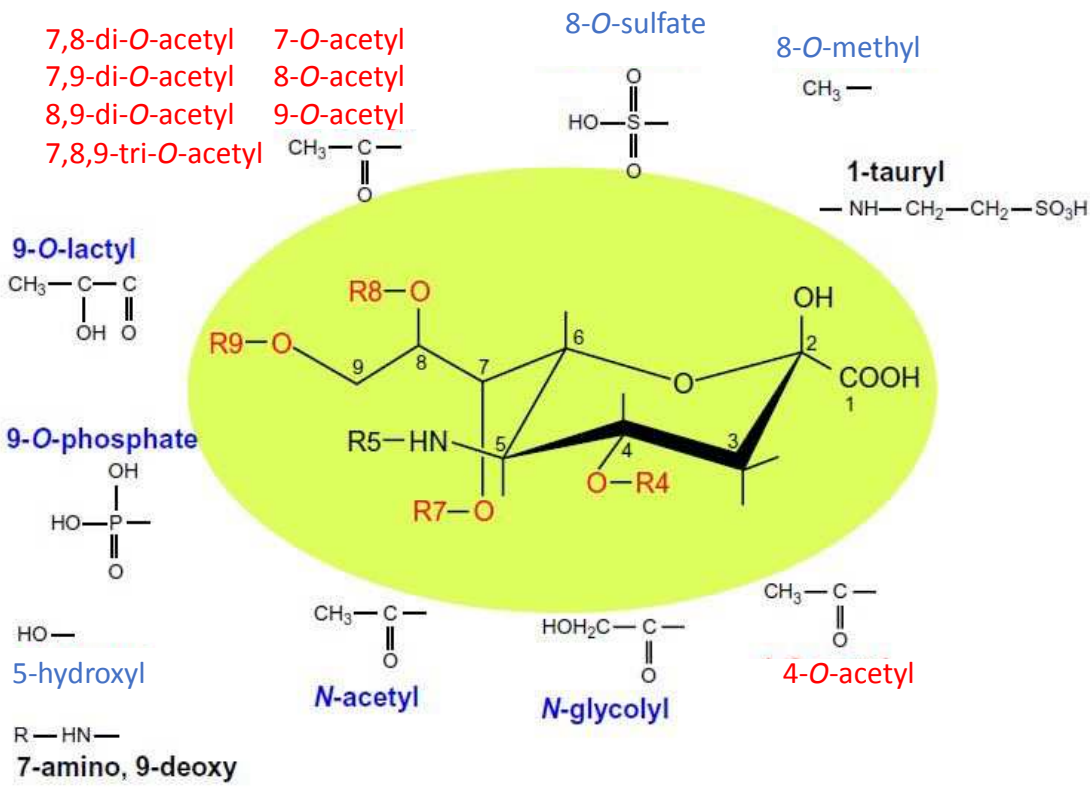


Figure 1.4 The family of naturally occurring sialic acids

1.2.6 Role of Sialic Acids in Cancer

Cancer is one of the main diseases in humans that have been reported to exhibit aberrant sialylation (Pietrobono and Stecca, 2021). There is either an overexpression and/or altered activity of sialyltransferases, an increased influx of metabolic substrates into the Sia synthesis pathway or a decreased synthesis of sialidases that catalyze the removal of Sia from glycan chains. All three mechanisms ultimately lead to an overexpression of Sia in cancer cells, contributing to a poor prognosis of the disease. Ras and c-Myc, are two

main proto-oncogenes that are involved in tumorigenesis and have also been proven to control transcription of sialyltransferases ST6Gal I and ST3Gal I, II and IV respectively (Seales *et al*., 2003; Sakuma *et al*., 2012). Ras specifically increases the α2,6-sialylation of ß1-integrin and facilitate the motility of the tumor cell, thus improving its chances of metastasizing (Seales *et al*., 2003). c-Myc also increases tumor cell motility but does this by increasing the expression of $sLe^{x/a}$ antigens (Sakuma *et al*., 2012). Furthermore, hypoxic conditions have been shown to induce the expression of ST3Gal I and subsequently $sLe^{x/a}$ antigens in colon cancer cells. The increased expression of this glycan antigen causes a favorable binding to selectins and entry of these tumor cells into the blood stream. There is also evidence of the role of sialidases in cancer metastasis. Colon cancer cells tend to downregulate the sialidases NEU1 and NEU4 to ensure hypersialylation of the laminin receptor ß4-integrin and a reduced hydrolysis of sLe^{x} antigens. This phenotype leads to an enhanced signaling of the laminin receptor and improves the ability of the cells to metastasize (Uemura *et al*., 2009; Shiozaki *et al*., 2011).

Sia specifically plays several key roles in the survival of cancer cells. Hypersialylation in tumor has been observed to help cancer cells evade cell death. One mechanism by which tumor cells undergo apoptosis is via the Fas receptor (FasR)-Fas ligand (FasL) pathway. The binding of the FasL to the FasR ideally triggers apoptosis and eventual cell death of cells, a pathway that has been widely studied. However, certain tumors have developed the ability to silence this pathway even when the FasL and FasR is present. Swindall and Bellis observed that, colon cancer cells overexpressing ST6Gal I, have an increased α2,6-sialylation of the FasR, a conformation that hinders the binding of the Fas-associated adaptor molecule (FADD) to the death domain of the FasR which

subsequently prevents the initiation of the death inducing signaling complex (DISC) and an ultimate shut down of the FasR-FasL apoptotic pathway (Swindall and Bellis, 2011).

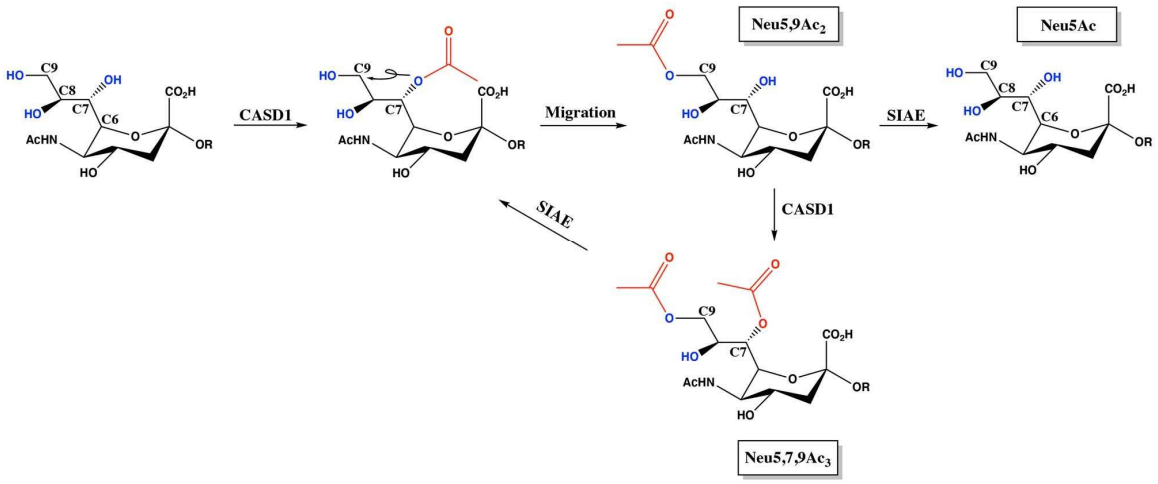
Sia also confers some level of resistance to cancer therapy when overexpressed in cells. Schultz et al. observed that an overexpression of Sia made tumor cells much more resistant to cisplatin, the platinum based chemotherapeutic drug. The group showed that when ST6Gal I is knocked out from tumor cells, the cells become overly sensitive to the drug and went further to demonstrate an increased expression of ST6Gal I in cisplatin-resistant ovarian tumor cells compared to non-resistant cells (Schultz *et al*., 2013). A similar resistance to the plant alkaloid and topoisomerase I inhibitor, irinocetan, has been observed in cancer stem cells when ST6Gal I is overexpressed (Swindall *et al*., 2013).

The action of sialyltransferases ultimately increases sialylation of glycan containing biomolecules. Sia overexpression in tumor generally fuels tumorigenesis and increases tumor survival, metastasis, and resistance to therapy as such, Sia metabolism holds the potential for improving cancer treatment if the enzymes involved in this mechanism are targeted.

1.2.7 Sia O-Acetylation And Deacetylation Is Catalyzed By CASD1 And SIAE

Aside from the level of Sia on cell surfaces, the nature of the Sia can also contribute to the overall physiology and role of Sia. Acetylation and deacetylation of 9-O and 7,9-O position of Sia is regulated by sialate-O-acetyltransferase (CASD1) and sialate-O-acetylesterase (SIAE) respectively. CASD1 adds acetyl functional groups to position 7-O of Sia, using acetyl CoA as the carbon source (Baumann *et al*., 2015; Orizio *et al*., 2015). This occurs in the Golgi apparatus during the post-translational modification of Sia-capped

glycoproteins (Baumann *et al*., 2015). Under physiological conditions the acetyl group can migrate from position 7 to position 8 or 9 of the Sia residue (Baumann *et al*., 2015).



Scheme 2.1 Sia acetylation scheme showing 7-O and 9-O acetylation and deacetylation catalyzed by CASD1 and SIAE

SIAE is a serine esterase that catalyzes the removal of acetyl functional groups from position 7 and 9 of Sia. This enzyme exists as two isoforms, the cytosolic Cse and the lysosomal Lse. The Lse isoform has been found to be mainly excreted out of the cells and associated with the cell membrane or in the culture media. The Lse isoform performs deacetylation of terminal Sia residues of membrane associated glycoproteins and glycolipids. The Cse isoform is known to be responsible for regenerating the native non-modified Sia from cytosolic acetylated Sia (Orizio *et al*., 2015). This ensures nonmodified Sia is available for the CMP-Neu5Ac synthase and other sialyltransferases to utilize these recycled Sia for other purposes. One other contrasting roles of the Cse and Lse is that Lse cleaves O-acetyl groups strictly from the 9-position while Cse can cleave from position 4 in addition to 9 (Butor *et al*., 1993; Takematsu *et al*., 1999).

1.2.8 Sialic Acid-Binding Immunoglobulin-Like Lectins (Siglecs) Are Inhibitory Receptors

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are a unique subset of the immunoglobulin-type (I-type) lectins and have the ability to bind to sialic acids on cell surfaces. Generally, the I-type lectins showcase a wide recognition function in binding to diverse glycan molecules, and Siglecs have been the most functionally and structurally studied subclass in this category (Powell and Varki, 1995; Angata 2002). There are 14 different Siglecs in humans that have been structurally classified so far. These have been divided into two main subfamilies based on the similarity in their structural sequence and their evolutionary conservation over time (Crocker and Varki, 2001). Four of the 14 Siglecs, namely Siglec-1 (sialoadhesin or CD169), Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein) and Siglec-15 share about 25 to 30% sequence similarity and have orthologues in all mammalian species (Crocker *et al*., 2007). The remaining 10 Siglecs namely Siglec-3 (CD33) and its related Siglecs (5-11, 14, 16) also share about 50 to 99% sequence identity. However, these have been reported to show some form of rapid evolution via gene duplication, exon loss or exon shuffling creating key differences in CD33-related Siglecs within mammals (Angata *et al*., 2004). Siglecs also have a unique expression and distribution on immune cells, and this ensures that their roles in specific immune cells are restricted to that cell alone. For instance, Siglec-1 is expressed mainly on macrophages but can be strongly induced on monocytes and monocyte-derived dendritic cells in vitro by culturing cells with type I interferons (Crocker and Varki, 2001). CD22 also found highly expressed on B cells is developmentally regulated and is expressed approximately at the time of Ig gene rearrangement. CD22 is a well-known modulator of

B-cell activation, primarily increasing the activation threshold needed for B-cell activation via the B cell receptor (BCR) (Tedder *et al*., 2005). It is does this by engaging Sia residues on the BCR via a cis-interaction and ultimately inhibiting the B-cell (Doody *et al*., 1995). However, CD22 is lost when B cells differentiate into plasma cells.

1.2.9 Structure And Role Of Siglecs

Siglecs have an amino-terminal V-set immunoglobulin domain that binds Sia and varying numbers of a C2-set immunoglobulin domain depending on the type of Siglec. The C2-set domain project the V-set domain away from the plasma membrane. They are type 1 membrane proteins and are found on various immune cells, playing primarily an immune-modulatory role. Several Siglecs have one or more tyrosine-based signaling motifs in their cytoplasmic tails called the immunoreceptor tyrosine-based inhibitory motif (ITIM). Those that do not constitutively contain ITIM regions may associate with membrane adaptor proteins that contain cytosolic tyrosine motifs. The ITIM region is consensus sequence of amino acids (V/I/L)XYXX(L/V), where X could be any amino acid. On the other hand, some Siglecs, notably Siglecs-14, -15, and -16 can associate with the DAP12(DNAX activation protein-12) ITAM-containing adaptor, thus mediating activating functions (Pillai *et al*., 2013).

Signaling via Siglecs begin with the binding of Sia to the V-set domain on the extracellular plasma membrane and a subsequent phosphorylation of the tyrosine residues in the ITIM domain by Src family kinases. The phosphorylated tyrosine residues recruit and activate SH2-domain-containing effectors, particularly the tyrosine phosphatases SHP-1 and SHP-2. Activated SHP-1 and SHP-2 phosphatases catalyzes the dephosphorylation

of prior-activated downstream proteins, counteracting any previously activating signals, and inhibiting the activation of the cells (Taylor *et al*., 1999).

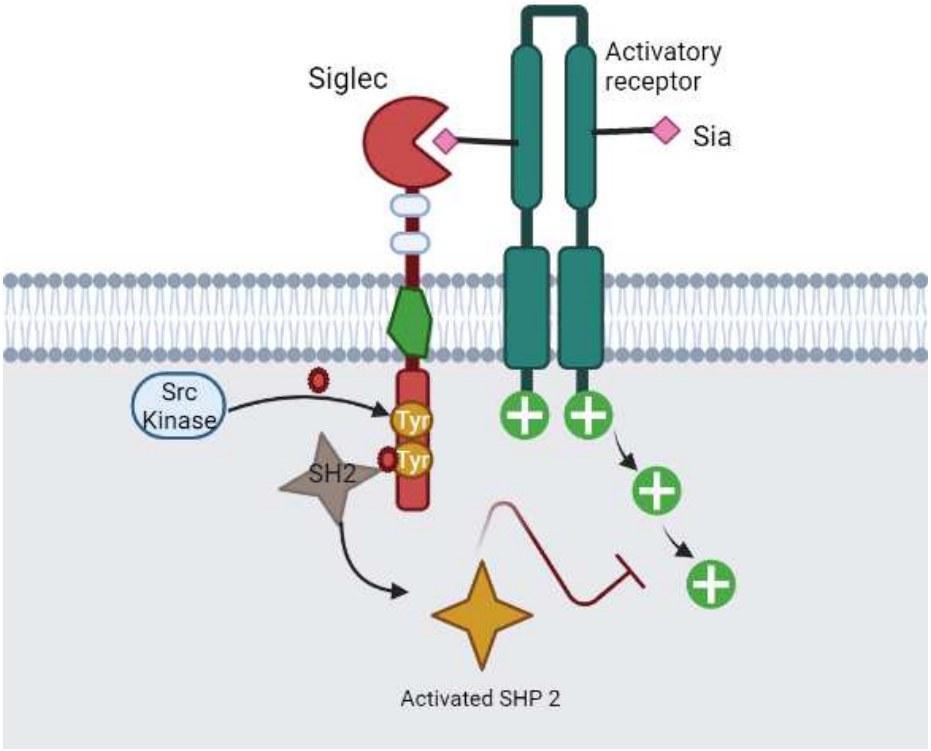


Figure 1.5 Sia-Siglec inhibitory signaling mechanism, showing binding of Sia to Siglecs and stimulation of downstream inhibitory proteins that acts to shut down activation signals.

1.2.10 Sia-O-Acetylation In Immune System Biology

Synthesis of O-acetylated Sia was first discovered by Corfield et al in 1976 when they observed N-acetyl-9(or 7)-O-acetyltransferase activity in a microsome fraction and in the cytosol of bovine submandibular glands (Corfied *et al*., 1976). Subsequently further evidence of O-acetylated sialic acids was reported by Hueso et al. in 1988 when they discovered the presence of a tentatively designated O-acetylated sialic acid in lymphocytes of spleen. Hueso's team set out to determine the sialic acid content of gangliosides from pig spleen lymphocytes and realized that the cellulose plates they used had two additional

molecules in addition to N-glycolylneuraminic acid and N-acetyleuraminic acid. One of these additional molecules was proposed to be an O-acetylated derivative of the disialoganglioside GD3, since after de-O-acetylation it co-migrated with GD3.

Hematopoiesis is a process where new immune cells and erythrocytes are generated. This primarily occurs in the bone marrow. The bone marrow also serves as a resident site for mature myeloid and lymphoid cells (Punt, 2019). These include antibody-secreting B cells (plasma cells) as well as some mature T cells. As such it is one of the most important tissues in the body, primarily also serving as a site for generation and storage of stem cells. In fact, the fate of hematopoietic progenitors has been shown to be partly determined by their surface levels of 9-O-acetyl sialic acids, specifically, plasmacytoid dendritic cells being the bone marrow progenitors with the lowest levels of 9-O-acetylated Sia (Netravali *et al*., 2019).

Though all lymphocytes are produced in the bone marrow, not all of them mature there, unlike B lymphocytes, T lymphocytes do not complete their maturation in the bone marrow. Instead, T lymphocytes travel to the thymus, which is another unique primary lymphoid organ to complete their maturation. T lymphocytes produced in the bone marrow have been observed to undergo a CDw 60 antigen differentiation in the thymus, that leads to the detection of a subpopulation of 9-O-acetylated GD3 gangliosides in the T lymphocytes population (Kniep *et al*., 1992). 9-O-acetylation has also been observed to play other key roles in the maturation of T lymphocytes in the thymus. Krishna and Varki (1997) demonstrated the preferential expression of 9-O-acetylated Sia on CD4 T cell lineage in normal B10 mouse lymphoid organ. This high degree of 9-*O*-acetylation is present on 90–95% of peripheral spleen and lymph node CD4 T cells. In contrast, only a

small minority of CD8 T cells and B cells show such levels of 9-*O*-acetylation (Krishna and Varki, 1997). The pattern of expression of Sia 9-O-acetylation was seen to markedly increase as cells matured from CD4/CD8 double positive (DP) and CD8 Single Positive (SP) to CD4 SP T lymphocytes suggesting a major role of this modified Sia in T lymphocyte maturation (Krishna and Varki, 1997).

The ability of influenza C virus spike glycoprotein Hemaglutinnin-Esterase-Fusion (HEF) to bind specifically to 9-O-acetylated Sia has provided a lot of possibilities in terms of isolating and quantifying 9-O-acetylated Sia containing biomolecules. Previously, it was believed that 9-O-acetylated Sia were restricted to gangliosides but this was proven to be untrue when Sia acetylation was detected in sialoglycoproteins prepared from blood leucocyte (Zimmer *et al*., 1994). Zimmer *et al*. further proved that, not only are these acetylated Sia present on sialoglycoproteins, their presence are not restricted to a specific leucocyte subpopulations, both B and T lymphocytes expressed these modified Sia. 9-O-acetylated Sia play key roles in the development of the cells of the adaptive immune system.

In rat erythrocytes the reduction in O-acetylation of Sia on cell surface glycoconjugates improved binding of erythrocytes to peritoneal macrophages (Kiehne and Schauer, 1992). This de-O-acetylation of Sia was thought to be catalyzed by an enzyme that had the same heat inactivation profile and inhibition profile as the non-specific esterase D. Varki *et al*. (1986) successfully isolated this enzyme from human erythrocytes, via several purification steps that also led to the co-purification of esterase D with the sialic acid-specific-O-acetylesterase. The Sialic acid-specific O acetylesterase was proposed to

be involved in recycling of O-acetylated Sias and was readily available in the cytosol of erythrocytes (Varki *et al*., 1986).

In modulating the immune system, Sia has been shown to be one of the key evolutionary mechanisms developed by pathogens and tumors to evade immune detection and/or activation. One of such mechanisms has been observed in all nine serotypes of the Group B *Streptococcus* (GBS). Group B *Streptococcus* (GBS) is known to cause bacterial pneumonia, sepsis, and meningitis in human newborns and is also recognized as a pathogen in adult populations, including diabetics, pregnant women, and the elderly (Carlin *et al*., 2007). The presence of Sia as terminal sugar residues on GBS serves as a molecular mimicry with which it is able to engage Siglec 9 and Siglec 5 on neutrophils and monocytes, limiting the activation of an effective innate immune response (Carlin *et al*., 2007). However, modification of the GBS Sia by O-acetylation leads to a reduction in the bacteria's ability to engage Siglecs on immune cells, with the degree of O-acetylation markedly affecting this interaction (Carlin *et al*., 2007). O-acetylation of terminal Sia of GBS could possibly be one of the ways of crippling the defense mechanism of this bacteria and making it more susceptible to host immune system. *Leishmania donovani* is a human blood parasite responsible for visceral leishmaniasis. Comparing virulent and avirulent *Leishmania donovani*, Ghoshal *et al*. (2009) noticed an increase in 9-O-acetylation of sialoglycoproteins in the virulent strain compared to the avirulent strain. The changes in acetylation levels conferred a 9 and 14.5 fold increase in infectivity and phagocytic index on the virulent strain and increased its entry into macrophages. Surprisingly, de-O-acetylation of the virulent strain led to a 3-fold decrease in its phagocytic index, confirming the important role of 9-O-acetylated sialoglycoproteins in the ability of this parasite to

cause diseases. Further studies performed one year later revealed a decrease in macrophage derived nitric oxide, interleukin-12 and interferon-gamma from macrophages infected with 9-O-acetylated sialoglycoproteins of this parasite *Leishmania donovani*, suggesting a suppression of leishmanicidal host immune responses.

The Sia-O-acetylation profile of tumors also contribute to the survival and overall physiology of the tumor. Acute lymphoblastic leukemia (ALL) has been linked with an increase in the 9-O-acetylation of Sia residues on lymphoblasts. Interestingly the serum of these patients was noticed to contain IgG1 and IgG2 antibodies raised against O-acetylated derivatives of Sia. The specificity of these antibodies for the acetylated Sia was markedly high and was completely abrogated when de-O-acetylated, confirming the specificity of the antibodies for the O-acetylated Sia variants. Further studies on this revealed that an increased Sia-acetylation in the peripheral blood mononuclear cells of both B- and T-ALL patients warranted an increase in antibody production and binding. The ability of these antibodies to bind acetylated Sia variants and trigger complement dependent cytolysis conferred some form of passive protection against ALL whiles creating a novel serodiagnostic assay via the specific antibody levels (Pal *et al*., 2000). In murine erythroleukemic cells (MEL), an increase in cell surface 9-O-acetyl groups of Sia caused by nocodazole treatment correlates with increased sensitivity to alternative pathway lysis. Thus, the addition of the 9-O-acetyl groups to Sia directly blocks the binding of human complement factor H (a negative regulator of the alternative complement pathway), abrogating its normal function in restricting alternative pathway activation (Shi *et al*., 1996).

Also in resting B-cells, the phosphoprotein receptor CD22 beta, which is a Sia binding inhibitory receptor was shown to have a reduced binding to Sia when the sugar is 9-O-acetylated. The presence of 9-O-acetylation on Sia was determined to mask the inhibitory role of Sia on B-cells, providing a means of regulating B-cell activity (Sjoberg *et al*., 1994). In lymph node-associated dendritic cells, all the cell surface CD22 beta ligands are predominantly masked by 9-O acetylation, suggesting that 9-O acetylated masking of Sia may regulate interactions between CD22 beta positive B cells and dendritic cells (Sjoberg *et al*. 1994). In addition to this, CD22's role in B cell receptor signaling has been demonstrated in vivo using mice that had mutations in the sialate-O-acetyl esterase gene. The impairment in de-O-acetylation of a 2-6-linked sialic acid resulted in enhanced BCR activation, defects in peripheral B cell development, and development of antichromatin autoantibodies and glomerular immune complex deposits, thus highlighting further the importance of 9-O-acetylated Sia in maintaining immunological tolerance in B cell lineage (Cariappa *et al*., 2008). In the human population, a strong association between defective SIAE alleles and rheumatoid arthritis and type 1 diabetes autoimmune disorders was observed in a preliminary study by Surolia *et al*. (2010). Considering the crucial role both SIAE and CASD1 play in the immune system, their expression needs to be properly coordinated. The dysregulation of these two genes in the immune system leads to one or several disease phenotypes. This type of dysregulation can render some survival advantage to tumors, leading to poor disease prognosis for patients. In this study, the exact role of Sia acetylation/deacetylation in lung and colon cancers was studied.

Given the role of Sia-O-deacetylation in modulating the immune cells via Siglec recruitment in B-cells, this study was designed to investigate how this functional group

affects Natural Killer (NK) cells' ability to identify and kill tumors. It was hypothesized that deacetylated Sias on cancer cell surfaces will engage Siglecs and dampen the immune response of NK cells. This will impede the ability of NK cells to reach their activating thresholds to stimulate any cytotoxicity. However, with the addition of acetyl groups to 9-O and 7,9-O positions of Sia, we hypothesize that Siglec binding will reduce, thus reducing the inhibitory signaling on NK cells and reducing their activating threshold needed to stimulate cytotoxicity.

1.3 Materials and Methods

1.3.1 Chemicals and Reagents

Human Siglec Fc Chimera Protein (7, 9, 10 and 11) were obtained from R&D Systems (Minneapolis, MN). The TransIT-LT1 transfection kit was obtained from Mirus Bio LLC (Madison, WI). RIPA Lysis and Extraction Buffer as well as Pierce Protease and Phosphatase Inhibitor were obtained from Thermo Fisher (Waltham, MA). Prolong Antifade-Gold with DAPI was obtained from Invitrogen (Waltham, MA). Carbo-Free Blocking Solution was also obtained from Vector Laboratories, (Burlingame, CA) while the CellTracker Green CFMDA dye was from ThermoFisher (Waltham, MA). SYTOX Red Dead Cell Stain and neuraminidase were obtained from Invitrogen (Waltham, MA) and Sigma Aldrich, (St. Louis, MO) respectively.

1.3.2 Cell Lines and Cell culture

A549 were grown in Dulbecco modified Eagle medium (DMEM Corning) with 10% fetal bovine serum (Corning) and 1% pen/strep (Cytiva Hyclone). HCT 116 cells were grown in RPMI 1640 medium (Corning) with 10% fetal bovine serum and 1% pen/strep. All cell lines were originally purchased from American Type Culture Collection. Concerning the passaging of cells, Cell Dissociation Buffer (Gibco,Waltham, MA) was used exclusively. In the generation of CRISPRi-mediated knockout of CASD1 and SIAE knockout HCT116 and A549 cell lines, the CASD1 and SIAE knockout HCT 116 and A549 cell lines were obtained from the group of Colin Parrish (Cornell University). CRISPR-Cas9 editing of CASD1 and SIAE in HCT116 and A549 cells was previously published (Barnard *et al*., 2019). In brief, for CASD1 knockout cells, paired Cas9 plasmids

targeting adjacent sites in early exons of CASD1 were transfected using TransIT-X2 (Mirus Bio LLC, Madison, WI). Transfected cells were selected with puromycin and single cell clones screened with PToV-P4 HE-Fc and sequence verified to confirm loss of CASD1 function in both alleles (Langereis *et al*., 2015). SIAE knockout cells were prepared using the same method with single cell clones screened by direct PCR amplification of the Cas9 targeted exons. Full sequencing of each allele and qPCR were performed to confirm deletion of the SIAE gene. A protein quantification and expression level of CASD1 and SIAE were previously analyzed and published (Grabenstein *et al*., 2021).

1.3.2.1 Generation Of CRISPR-Cas9 Mediated Knockout Of HCT 116 Cell Lines

Cas9 was lentiviraly transduced and constitutively expressed in HCT 116 cells by the Bassik lab (Stanford University, Stanford, CA). These CRISPR-Cas9 stably expressing cells and the third-generation lentiviral system were gifted from the Bassik lab. To generate CRISPR knock out cell lines, targeted guide sequences, with the highest predicted CRISPR activity scores reported by (Horlbeck et al. 2016), were cloned into puro-resistant, mCherry expressing, lentiviral vector pMCB320. (Han et al. 2017) These plasmids, along with components of the third-generation lentiviral systems, were transfected into HEK293T cells using TransIT-LT1 (Mirus Bio LLC, Madison, WI). The resulting lentiviruses were then infected into the HCT 116 Cas9- expressing cells. Transduced cells were selected with puromycin, and single cell sorted using mCherry expression. Knock-out efficiencies were confirmed in triplicate by RT-qPCR amplification and western blot analysis.

The protospacer sequence for SIAE sgRNA:

5^{r}- CACCGGACCTTGGCGCAGGGTCA -3^{r}

The protospacer sequence for CASD1 sgRNA: 5^{r}-

CACCGGCAATATGTTTATCTACA −3r

The primer sequences for SIAE:

FW: 5r- ACCTTCCCTTACCACCCAGT-3r

RV: 5- CCGATGATGCTTCCCTCAGT-3r

The primer sequences for CASD1:

FW: 5r- AAGACTCTTGCTCTGGCACC-3r RV: 5-

TGCCAATGTCTTAGCCACCA-3r

1.3.2.2 Western Blot Analysis

Cells were harvested using Cell Dissociation Buffer, enzyme free and PBS (Gibco, Waltham, MA). Cells were then lysed with RIPA Lysis and Extraction Buffer (Thermo Fisher, Waltham, MA), according to the manufacturer's instruction. Pierce Protease and Phosphatase Inhibitor (Thermo Fisher, Waltham, MA) was added to the RIPA buffer, according to the manufacturer's instruction. Total protein concentration was measured using a BCA assay (Sigma Aldrich, St. Louis, MO). The plate was read using a BioTek Cytation Live Cell imager (BioTek, Winooski, VT). Media and cell extract samples were subjected to SDS– PAGE using 4–12% (w/v) Criterion TGX Precast Gel (BioRad). Subsequent transfer was conducted with a Trans-Blot Turbo RTA Midi 0.2 um nitrocellulose transfer kit and the associated Turbo-Blot Turbo Transfer System (BioRad). Membranes were blocked for 30 min in PBS containing 0.1% (v/v) Tween 20 (PBST) and 5% (w/v) BSA. Membranes were either subsequently incubated with Anti-SIAE antibody (Abcam) at a dilution of 1:1000 or Anti- CASD1 antibody (Sigma) at a

dilution of 1:5000. Both antibodies were reconstituted in Intercept (PBS) Protein Free Blocking buffer (Li- COR, Lincoln, NE), incubated at 4°C overnight with gentle shaking. The membranes were washed three times in PBS (Corning) containing 0.1% Tween-20 for 10 min each time and subsequently incubated in the dark with the appropriate secondary antibody. Goat Anti-mouse IRDye secondary antibody (Li-COR, Lincoln, NE) was utilized for detection of SIAE and Goat Anti-rabbit IRDye secondary (Li-COR, Lincoln, NE) was used for detection of CASD1. Incubation occurred for 1 h at a dilution of 1:10,000 for each in PBS containing 0.1% Tween-20. Membranes were analyzed using the Odyssey CLx Imager (Li-COR, Lincoln, NE).

1.3.2.3 Immunofluorescence Microscopy And Flow Cytometry

Cells were stained using probes derived from viral hemagglutinin esterase proteins fused to human IgG1 Fc (HE-Fc). The porcine torovirus strain 4 HE-Fc (PToV HE-Fc) primarily recognizes 9-O- Ac and the bovine coronavirus Mebus strain HE-Fc (BCoV HE-Fc) recognizes 7,9-O-Ac and shows low levels of binding to 9-O- Ac. For immunofluorescence microscopy, cells were seeded onto glass coverslips and incubated overnight at 37°C and 5% CO_2. Coverslips were fixed in 4% paraformaldehyde (PFA). Coverslips were incubated with Carbo-Free Blocking Solution (Vector Laboratories, Burlingame, CA), with optional permeabilization with 0.001% Tween-20. To stain, HE-Fc probes were pre-complexed with Alexa- 488 labeled anti-human IgG antibody then diluted in Carbo-Free blocking solution to a final concentration of 5 μg/mL HE-Fc and 1:500 of secondary antibody. Cells were stained with HE-Fc/anti-IgG complex and coverslips were mounted using Prolong Antifade-Gold with DAPI (Invitrogen, Waltham,

MA). Cells were imaged using a Nikon TE300 fluorescent microscope. For flow cytometry, cells were seeded onto nonadherent cell culture dishes and incubated overnight at 37°C and 5% CO_2. Cells were collected using ice-cold PBS to retain surface glycans, then fixed in 4% PFA. Cells were blocked as above and incubated with commercial lectins SNA and MAA II con- jugated to FITC (Vector Laboratories, Burlingame, CA). A Millipore Guava EasyCyte Plus flow cytometer (EMD Millipore, Billerica, MA) was used to collect data, analysis using FlowJo software (TreeStar, Ashland, OR). Statistical analyses were performed in PRISM software (GraphPad, version 8).

1.3.2.4 Quantification Of Sia Variants

The Sia composition of cells were determined by incubating with 2 M acetic acid at 80°C for 3 h, filtration through a Microcon 10- kD centrifugal filter (Millipore) and drying in a SpeedVac vacuum concentrator. Released Sia were derivatized with 1,2-diamino-4, 5- methylenedioxybenzene (DMB, Sigma Aldrich, St. Louis, MO) for 2.5 h at 50°C (Varki and Diaz, 1984). HPLC analysis was performed using a Dionex UltiMate 3000 system with an Acclaim C18 column (ThermoFisher) under isocratic elution in 7% methanol, 7% acetonitrile and 86% water. Sia standards included bovine submaxillary mucin and commercial standards for Neu5Ac and Neu5Gc (Sigma Aldrich, St. Louis, MO). Statistical analyses were performed in PRISM software (GraphPad, version 8).

1.3.3 Cell Proliferation Assays

1.3.3.1 NK Mediated Cytotoxicity Assay

Cell lines were grown and cultured accordingly. Control HCT 116, A549 and HEK

293 and ΔSIAE HCT 116, A549 and HEK were labelled and incubated with CellTracker Green CFMDA dye (ThermoFisher, Waltham, MA), according to the manufacturer's instruction. The stained cells were harvested using enzyme-free Cell Dissociation Buffer (Gibco, Waltham, MA), counted and aliquoted into 96 well v-bottom plates. Cells designated as controls were pretreated with 10-μM neuraminidase (Sigma Aldrich, St. Louis, MO) in DPBS for 1 h, centrifuged and resuspended. Cultured NK-92 (ATCC) cells were also grown and harvested accordingly, counted and co-incubated in a previously titrated ratio of effector:tumor cell (4:1 for HCT116, 10:1 for A549 and 4:1 for HEK 293 cells) for 4 h. After co-incubation, cells were centrifuged and washed. APC Annexin V and 7-Amino- Actinomycin D (7AAD) were aliquoted into designated wells according to the manufacturer's instruction. The cells were vortexed, incubated NK and then subjected to flow cytometric analysis utilizing using Accuri C6 Plus Flow Cytometer. After gating on CFMDA+ cells, cell viability was assessed by analyzing APC Annexin V and 7-AAD (Vermes *et al.,* 1995, Xiao *et al*., 2016).

1.3.3.2 Preparation Of Siglec-Fc Antibody Conjugates

A 4 mg/mL solution of anti-hFc secondary (Jackson Immuno, West Grove, PA Alexa Fluor® 488 AffiniPure Goat Anti-Human IgG, Fc*γ* fragment) was prepared in PBS (Corning) containing 0.5% bovine serum albumin (BSA). Human Siglec Fc Chimera Protein (7, 9, 10 and 11 from R&D Systems) was added to the anti-hFc secondary for a final concentration of 0.05 nM.

1.3.3.3 Siglec Binding Assay

Cells grown to 80% confluency were washed once with PBS and treated with Cell Dissociation Buffer (Gibco, Waltham, MA). Cells were centrifuged, counted and

resuspended with PBS (Corning) containing 0.5% bovine serum albumin (BSA Fishersci, Hampton, NH) into a sterile 96 well plate at a concentration of 2×10^5 cell per well. Cells were centrifuged and resuspended in Siglec-Fc antibody conjugates (prepared as previously described), incubated for 30 min at 4°C in the dark. Cells designated as controls were pretreated with 10-μM neuraminidase (Sigma Aldrich, St. Louis, MO) in DPBS for 1 h, prior to incubation, centrifuged and resuspended with Siglecs- Fc conjugates. Additionally, background controls were conducted by incubating cells in anti-hFc secondary (Jackson Immuno, West Grove, PA Alexa Fluor® 488 AffiniPure Goat Anti-Human IgG, Fc*γ* fragment) in PBS containing 0.5% BSA. Upon removal, cells were washed three times and resuspended in PBS containing 0.5% BSA. Cells were incubated for 15 min with 5 nM of SYTOX Red Dead Cell Stain (Invitrogen, Waltham, MA) prior to analysis by flow cytometry. All flow cytometry data was analyzed using FlowJo v. 10.0 (Tree Star, Ashland, OR). All experiments were performed in three technical and biological replicates.

1.4 Results And Discussion

1.4.1 Precluding And Re-establishing Siglec Binding In SIAE And CASD1 Knockout Cancer And Noncancerous Cell Lines

Aberrant glycosylation, hypersialylation and noncanonical Sias have all been documented on the cells from a variety of different cancers (renal, prostate, colon, breast, head and neck), and are proposed to modulate cancer progression and metastasis. Furthermore, hypersialylation in the tumor microenvironment is linked to immune suppression (Lubbers *et al*., 2018). Aside from what is currently known from the autoimmune literature, there is little known about how noncanonical Sias, such as the O-acetylated Sias, regulate the Sia-Siglec immune evasion pathway or what processes are involved (Varki *et al*., 2015)

SIAE could be utilized by cancers to decrease 9-O-acetylation levels via removal of acetyl groups. To determine if loss of SIAE and the increasing 9-O-acetylation in cells would decrease Siglec binding, the SIAE gene was knocked out by CRISPR mediated gene disruption. These studies were performed with cells from colon cancer (HCT 116), lung cancer (A549), as well as testing a noncancer-associated but well-established kidney cell line (HEK 293). The resulting knock out cell lines were screened for Siglec binding, along with a negative control of pre-treating cells with neuraminidase from Vibrio cholera to remove Sia and ablate Siglec binding. Compared with the control cell line, the ΔSIAE colon (Figure 1.6A-D), lung (Figure 1.7A-D) and kidney (Figure 1.8A-D) cell lines all showed decreased Siglec 7, 9, 10 and 11 binding.

The ΔSIAE HCT 116 cell line showed significant decreases in Siglec 7, 9 and 10 binding, as well as a mild reduction in Siglec 11 binding. ΔSIAE A549 cell lines displayed

a small decrease in the binding of all Siglecs when compared with the ΔSIAE HCT 116, while the noncancerous cell line HEK 293 was least affected, with very little Siglec 10 engagement seen.

As increase of 9-O-acetylated Sias through loss of SIAE resulted in decreased Siglec binding, we sought to determine if loss of all 9-O- acetyl modifications would result in an equivalent increase of Siglec binding. The CASD1 gene for the O-acetyltransferase was removed from HCT 116, A549 and HEK 293 cell lines using CRISPR-Cas9 gene editing. Siglec binding to ΔCASD1 HCT 116 (Fig 1.6E-H), and ΔCASD1 A549 cells (Fig. 1.7E-H), increased compared with that seen on control cells, and even more compared with ΔSIAE HCT 116 and ΔSIAE A549 cells. This supports the role for 9-O-acetylation levels in modulating Siglec engagement. Siglec 9 and 11 showed the greatest increase in binding over control cells for ΔCASD1 HCT 116 and ΔCASD1 A549 cells. HEK 293 cells were again least affected by gene modulation, with very little Siglec 10 engagement and no modulation after removal of CASD1 (Fig. 1.8E-H).

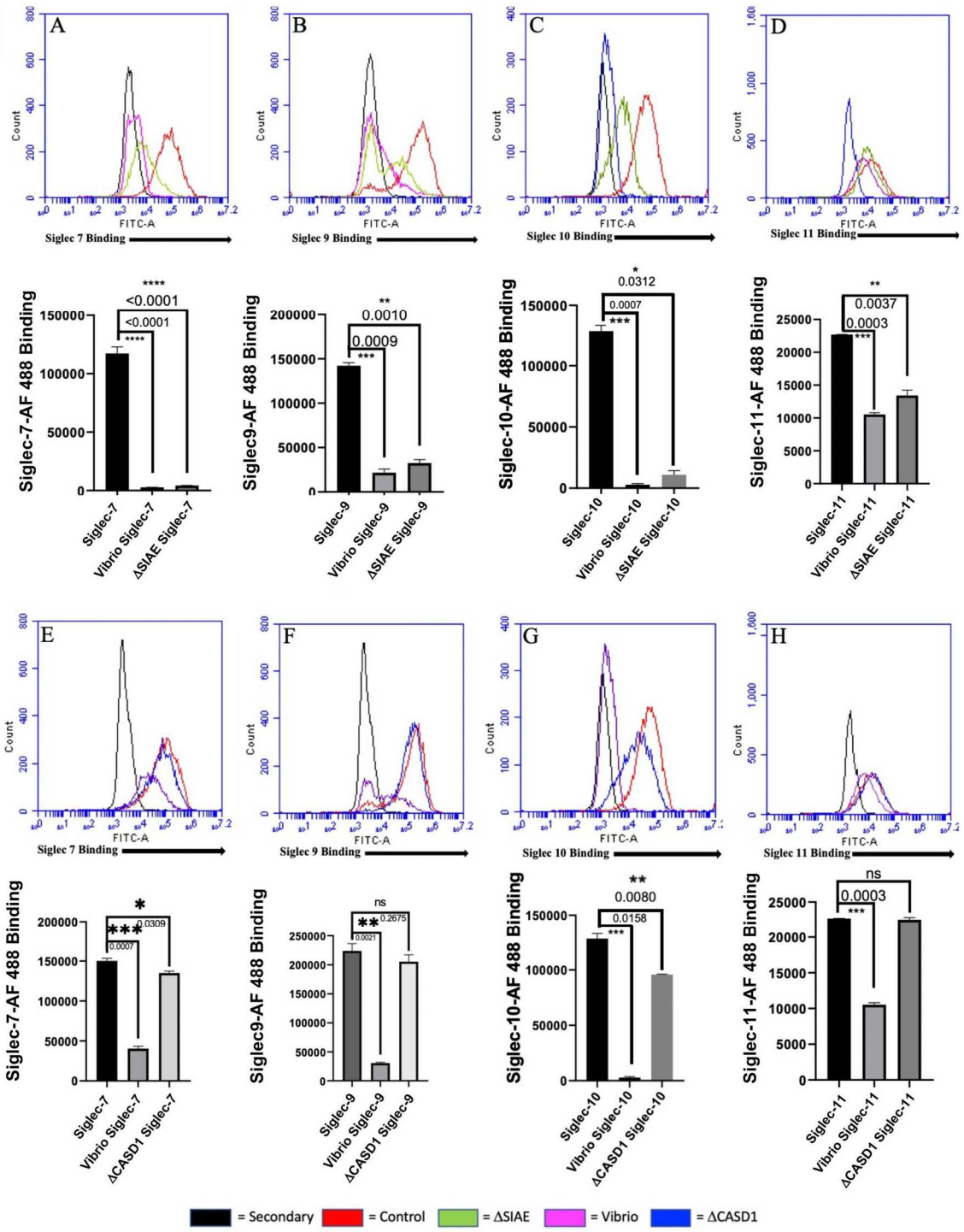


*Figure 1.6 Siglec assay on CRISPR Cas-9 gene edited colon cancer cell line HCT 116. Control HCT 116 cell lines, ΔSIAE HCT 116 and ΔCASD1 HCT 116 were incubated with commercially available Siglecs 7, 9, 10 and 11 (preconjugated to Alexa fluor 488) for 30 min at 4˚C. Control cell lines were incubated with neuraminidase from Vibrio Cholera for 1 h at 37˚C prior to Siglec incubation. (**A**–**D**) a reduction in Siglec binding resulted in ΔSIAE HCT 116 cell lines compared with control. (**E**–**H**) Siglec binding either increased in ΔCASD1 HCT 116 cells lines or was comparable to the control cell line. Error bars represent standard deviation of triplicate samples.*

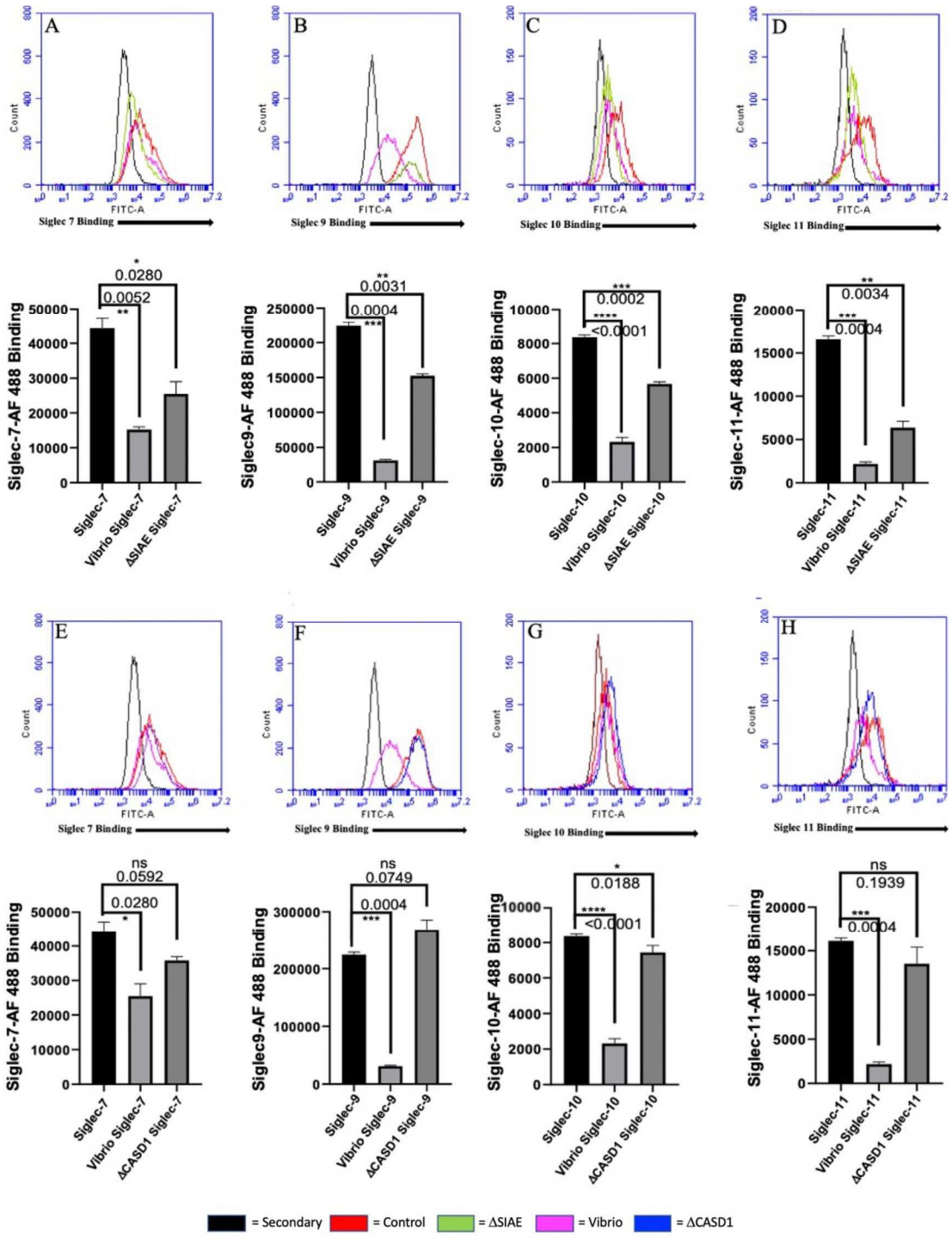


*Figure 1.7 Siglec assay on CRISPR Cas-9 gene edited lung cancer cell line A549. Control A459 cell lines, ΔSIAE A549 and ΔCASD1 A549 were incubated with commercially available Siglecs 7, 9, 10 and 11 (preconjugated to Alexa fluor 488) for 30 min at 4°C. Control cell lines were incubated with neuraminidase from Vibrio Cholera for 1 h at 37°C prior to Siglec incubation. (**A**–**D**) a reduction in Siglec binding resulted in ΔSIAE A549 cell lines compared with control. (**E**–**H**) Siglec binding in ΔCASD1 A549 cells lines was comparable to the control cell lines. ΔCASD1 A549 cells bound with a higher affinity to Siglec 9, however, compared with the control cell line. Error bars represent standard deviation of triplicate samples.*

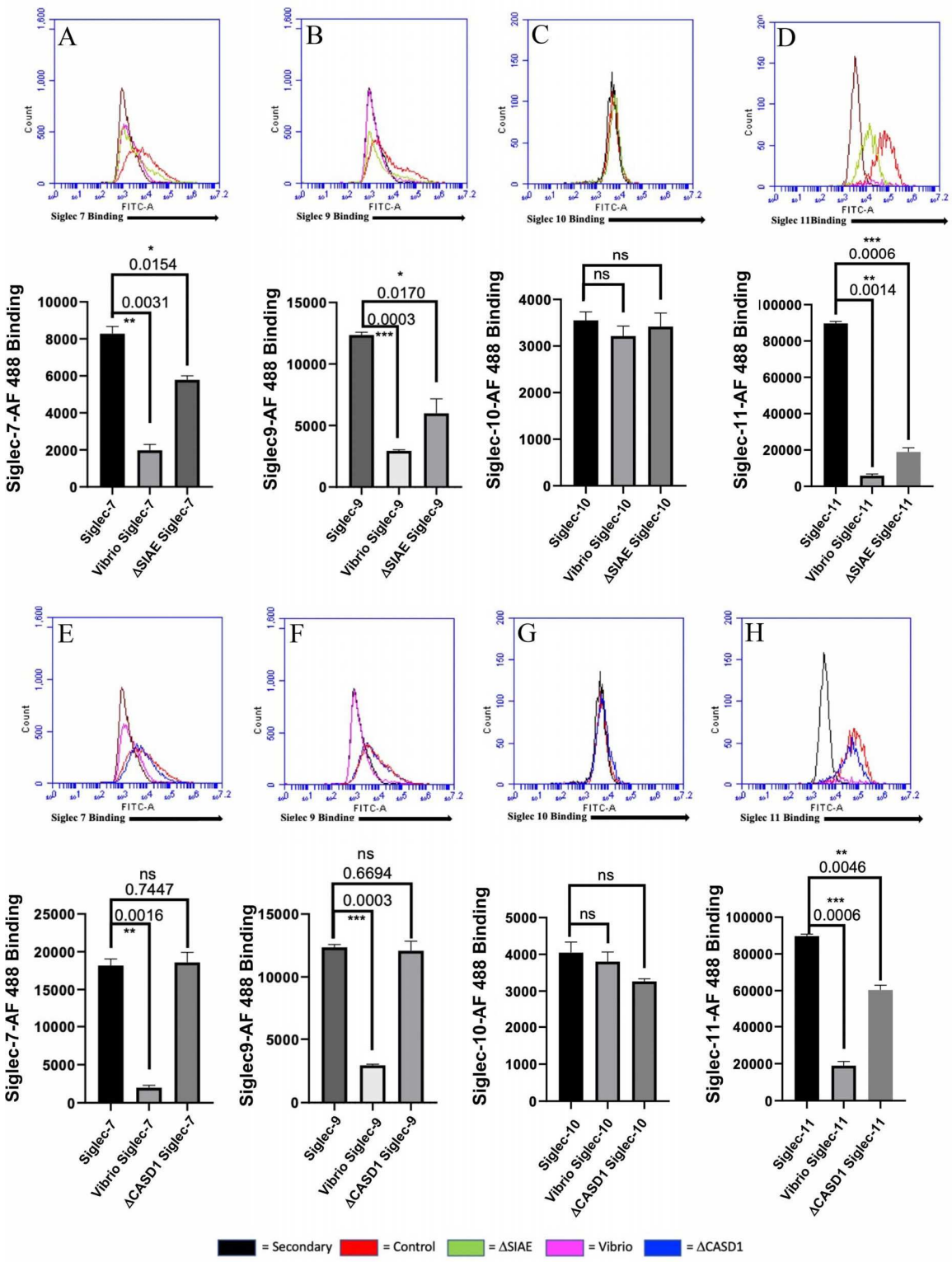


Fig. 1.8 Siglec assay on CRISPR Cas-9 gene edited cell line HEK 293. Control HEK 293 cell lines, ΔSIAE HEK 293 and ΔCASD1 HEK 293 were incubated with commercially available Siglecs 7, 9, 10 and 11 (preconjugated to Alexa fluor 488) for 30 min at 4◦C. Control cell lines were incubated with neuraminidase from Vibrio Cholera for 1 h at 37◦C prior to Siglec incubation. (A, B and D) a reduction in Siglec 7, 9 and 11 binding resulted in ΔSIAE HEK 293 cell lines compared with control, whereas no Siglec 10 engagement occurred (C). (E, F and H) Siglec binding was re-established in ΔCASD1 HEK 293 cells lines for 7, 9 and modestly in 11, while no change resulted in Siglec 10 (G). Error bars represent standard deviation of triplicate samples.

1.4.2 Expression Of 9-O-And 7,9-O-Acetyl Sia On HCT-116 Cell Variants

Virolectin probes were used to stain for the presence on 9-O-acetyl Sia (porcine torovirus, PTOV) and 7,9-O-acetyl Sia (bovine coronavirus, BCoV) as previously described (Langereis *et al*., 2015; Wasik *et al*., 2017; Barnard *et al*., 2019). HCT-116 cells showed the presence of low levels of 9-O-Ac internally (Fig. 1.9A-C), likely associated with the Golgi, as well as on the cell surface (Fig. 1.9J-L). Very little to no 7,9-O-Ac Sia staining was seen within the control cells (Fig. 1.10A-C) or on the cell surface (Fig. 1.10J-L). ⊿SIAE HCT 116 cells had increases of both internal and surface staining of 9-O- Ac (Fig. 1.9D-F and M-O) and 7,9-O-Ac (Fig. 1.10D-F and M-O when analyzed by fluorescent microscopy. Similar to previous reports for A549 and HEK 293 cells (Barnard *et al*., 2019), removal of CASD1 in colon cancer cell line HCT 116 in (⊿CASD1 cells) showed no binding of either 9-O-Ac (Fig. 1.9G-I and P-R) or 7,9-O-Ac Sia (Fig. 1.10G-I and P-R). HPLC analysis revealed relatively low levels of modified Sia, with 9-O-Ac making up <1% of the total Sia collected from cells. This is consistent with previously published data on expression of modified Sia for A549 and HEK 293, included in Table 1.1 (Barnard *et al*., 2019).

Table 1.1 Sialic acid distribution (%) in A549 and HEK 293 cells as determined by Bernard et al., 2019

Cell type	**Neu5Ac**	**Neu5Gc**	**Neu5,9Ac2**	**Neu5,7,9Ac2**
A549	96.40	2.21	1.39	ND
HEK 293	96.72	2.26	1.02	ND

Total sialic acid was collected from cells using acid hydrolysis and sialic acid forms were determined using HPLC analysis. Percentages show relative proportions of each sialic acid form collected from each cell type (Barnard et al. 2019)

No 7,9-O-Ac Sia staining was found using HPLC, consistent with immunofluorescence staining. HPLC did show that ⊿SIAE had higher levels of 9-O-Ac compared with the control HCT 116 cells (Fig. 1.9).

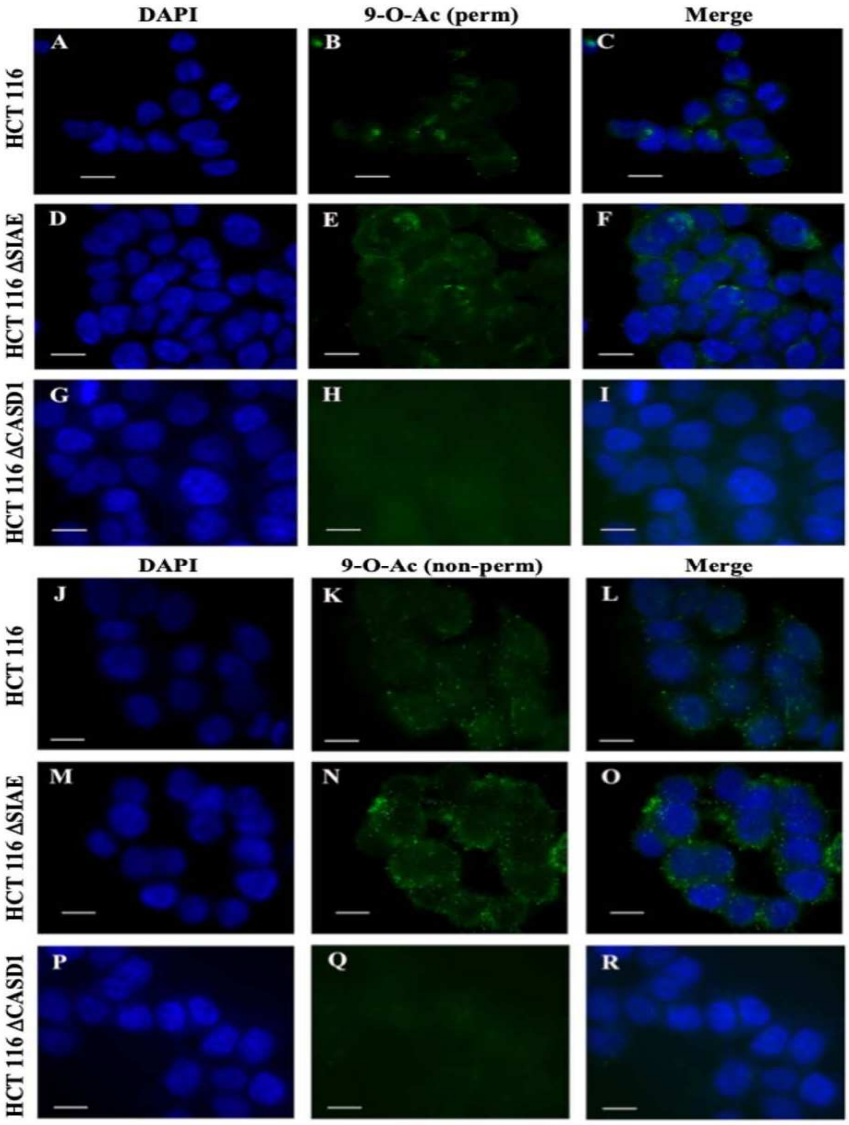
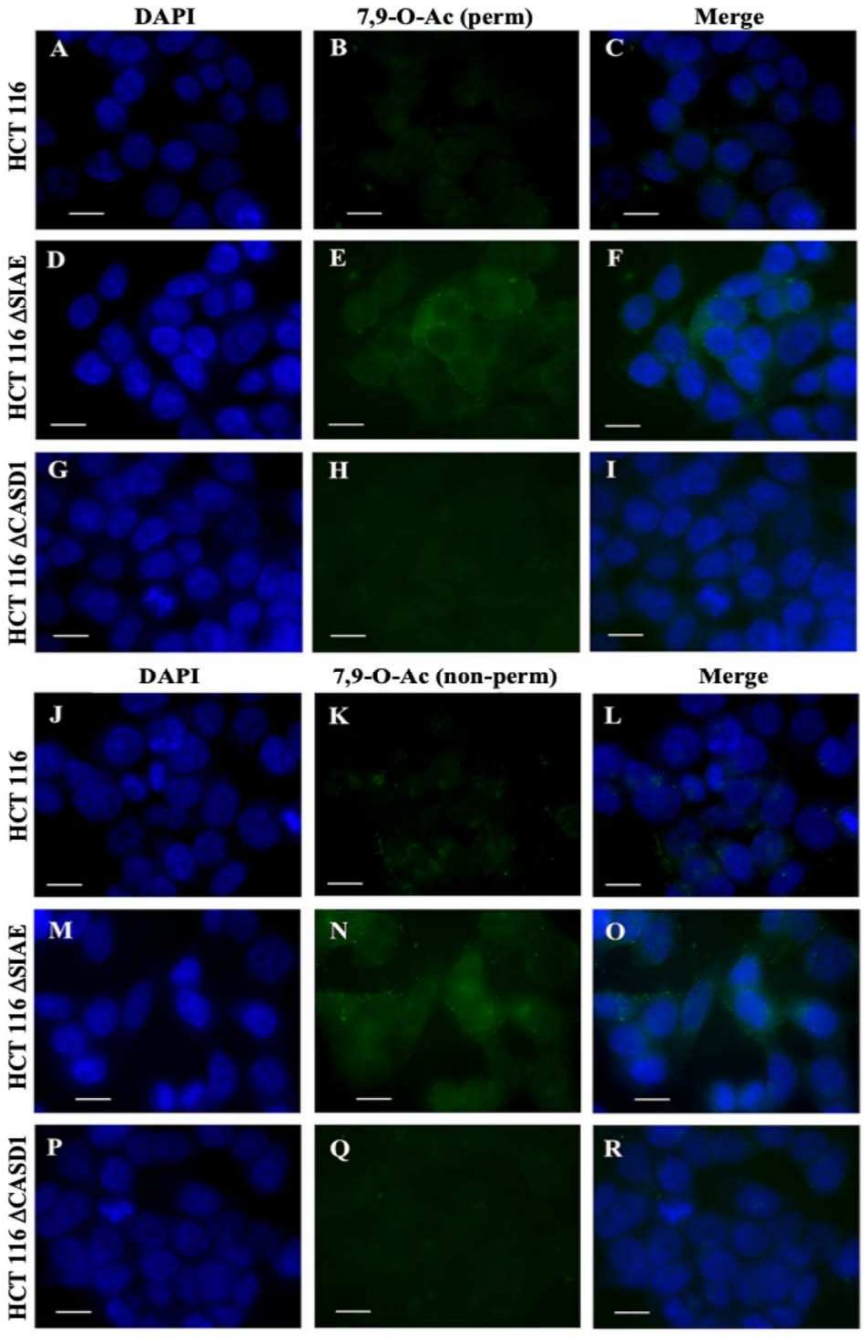


Fig. 1.9 Internal and surface distribution of 9-O-acetylated Sia on CRISPR Cas-9 gene edited cell line HCT 116. Cells were stained for the presence of 9-O-acetyl Sia with virolectin hemagglutinin esterases human IgG1 Fc (HE-Fc) probes for 1 h at 4°C (derived from bovine coronavirus, BCoV HE-Fc, that were pre- complexed to Alexa-Fluor 488). (A–C) Internal staining of HCT 116 WT cells resulted in traces amount of 7, 9-O-aceylation in the Golgi complex compared with HCT 116 OSIAE (D–F) with no acetylation seen in HCT 116 OCASD1 cell lines (G–I). Similarly, trace levels of 7,9-O-aceylation occurred in control on the surface (J–L), with increased amounts on the HCT 116 OSIAE, (M–O) and none on the HCT 116 OCASD1cell lines (P–R).


DAPI
7,9-O-Ac (perm)
Merge
HCT 116
HCT 116 ΔSIAE
HCT 116 ΔCASD1
A
B
C
D
E
F
G
H
I
DAPI
7,9-O-Ac (non-perm)
Merge
HCT 116
HCT 116 ΔSIAE
HCT 116 ΔCASD1
J
K
L
M
N
O
P
Q
R

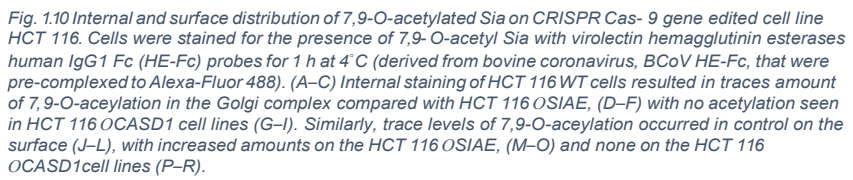


Fig. 1.10 Internal and surface distribution of 7,9-O-acetylated Sia on CRISPR Cas- 9 gene edited cell line HCT 116. Cells were stained for the presence of 7,9- O-acetyl Sia with virolectin hemagglutinin esterases human IgG1 Fc (HE-Fc) probes for 1 h at 4°C (derived from bovine coronavirus, BCoV HE-Fc, that were pre-complexed to Alexa-Fluor 488). (A–C) Internal staining of HCT 116 WT cells resulted in traces amount of 7, 9-O-aceylation in the Golgi complex compared with HCT 116 OSIAE, (D–F) with no acetylation seen in HCT 116 OCASD1 cell lines (G–I). Similarly, trace levels of 7,9-O-aceylation occurred in control on the surface (J–L), with increased amounts on the HCT 116 OSIAE, (M–O) and none on the HCT 116 OCASD1cell lines (P–R).

1.4.3 Determining How Alteration In 9-O And 7,9-O-Acetylation Modulates Siglec Binding Of Colon Cancer Cells Via The Removal Of 9-O And 7,9-O-Ac

To better understand how 9-O vs. 7,9-O-acetylation modulates Siglec binding, viral esterases were employed. Colon cancer cells were first treated with viral HE proteins that specifically target 9-O and 7,9-O-Ac on Sia. The porcine torovirus esterase (PToV HE-Fc) primarily recognizes and removes 9-O-Ac, whereas the bovine coronavirus Mebus esterase (BCoV HE-Fc) recognizes and removes 7,9-O- Ac (Langereis *et al*., 2015; Wasik *et al*., 2017; Barnard *et al*., 2019). After the esterase was removed the cells were incubated with Siglec- Alexa Fluor 488 conjugates and flow cytometry used to quantify binding. Removal of 9-O-and 7,9-O-acetyl functional groups resulted in increased binding to Siglecs 7, 9 and 10 compared with untreated controls (Fig. 1.11).

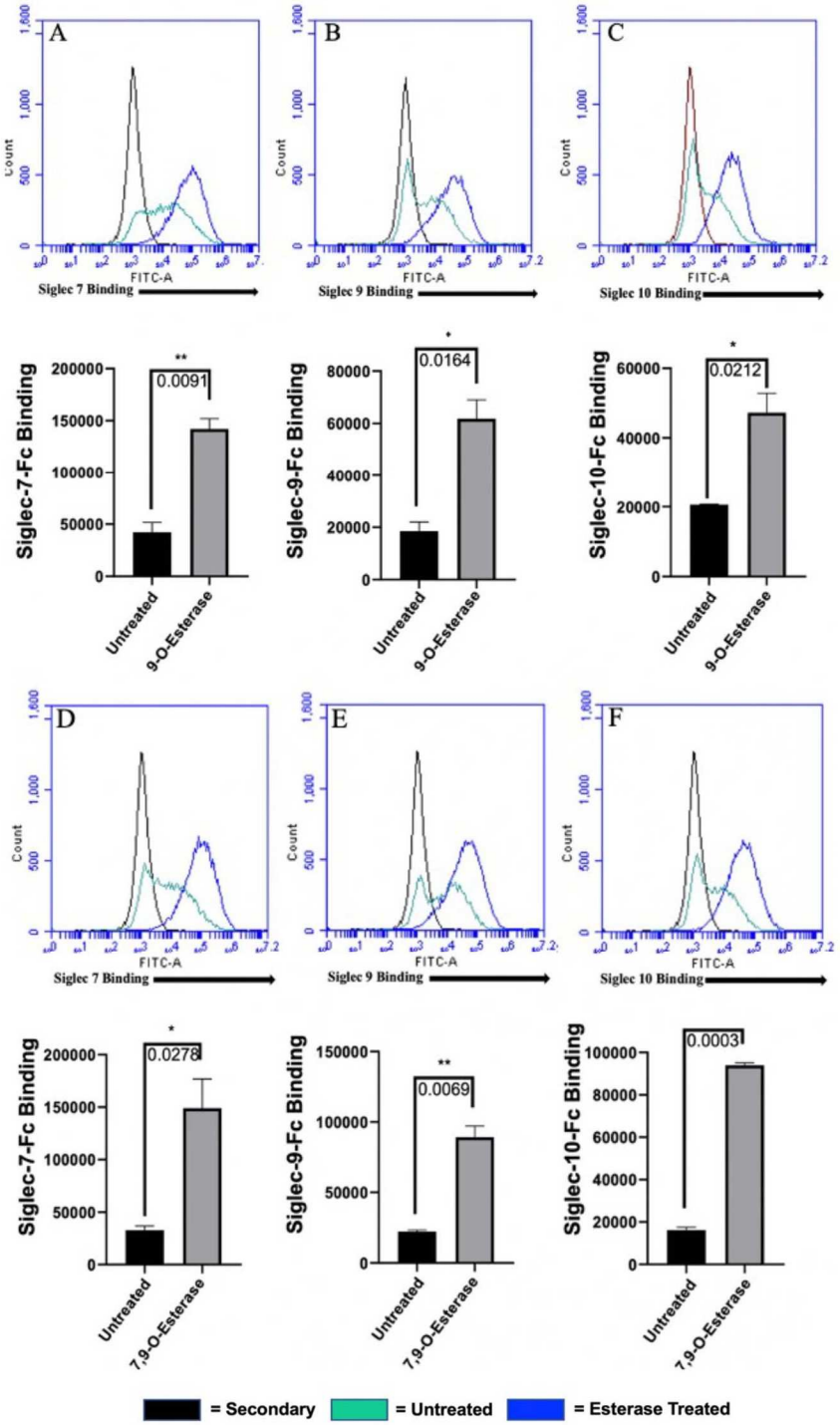


*Figure 1.11 The use of sialylglycan recognition probes (SGRP) to measure the level of 9-O and 7,9-O- acetylation on colon cancer cell line HCT 116. Cells were pre- treated with viral hemagglutinin esterase proteins that specifically target and remove either 9-O or 7,9-O-acetylation (porcine torovirus- PTOV HE and bovine coronavirus- BCoV HE, respectively). Cells were then washed and incubated with Siglecs 7, 9 and 10 that were pre-conjugated to Alex Fluor 488 (AF-488). In the absence of 9-O acetylation an increase in Siglecs 7, 9 and 10 binding resulted compared with untreated cells (**A**–**C**). Similarly, when cells were treated with BCoV HE an increase in Siglec binding also occurred indicating that both 9-O and 7-O- acetylation are involved in modulated Siglec binding (**D**–**F**). Error bars represent standard deviation of triplicate samples.*

1.4.4 Effects Of ⊿SIAE And ⊿CASD1 In HCT 116 On The Expression Of *α* 2–3 And *α* 2–6-Linked Sia

Elevated levels of cell-surface *α*2,6-linked Sias have been associated with metastasis and therapeutic failure in colorectal cancer (Park and Lee, 2013). *α*2, 6-linked Sias found on membrane-anchored protein receptors may also modulate adhesion, invasiveness and motility in colorectal cancer cells, which may influence the common metastasis to the liver or lung (Park and Lee, 2013). Previous research has identified sialyltransferases as a therapeutic target for regulating the overexpression of cell-surface *α*2,6-linked Sias in colorectal cancer patients. Staining of control, ⊿SIAE and ⊿CASD1 HCT 116 cells using standard lectins (MAL and SNA) revealed both *α*2–3 (Fig 1.12A-C) and *α*2–6-linked Sia (Fig 1.12J-L).

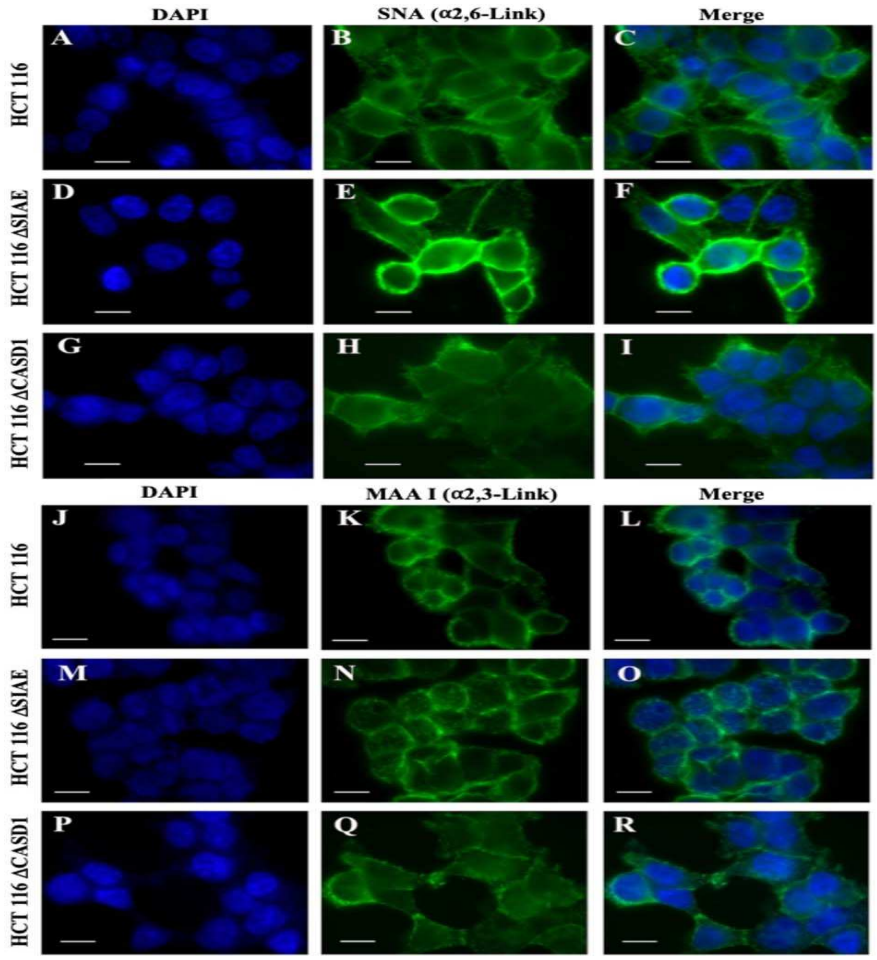


Figure 1.12 Determining the Linkage of Sia. Staining using standard plant lectins revealed the presence of α2–6-linked Sia, with higher levels than previously reported for most human cell lines. The highest levels are seen in the HCT 116 ΔSIAE cell lines (D–F) compared with both HCT 116 WT (A–C) and HCT 116 ΔCASD1 (G–I). α2–3-linked Sia was also present, evenly across cell lines, (J–L) HCT 116 WT, (M–O) HCT 116 dSAIE and (P–R) HCT 116 OCASD1.

In ΔSIAE cells lines, a substantial increase in lectin binding to α2–6-linked Sia was seen (Fig 1.12D-F and 8) but the ΔCASD1 cells showed no change in the amount of lectin staining for either α2–3 (Fig 1.12G-I) or α2–6- linked Sia (Fig 1.12P-R and Fig 1.13)

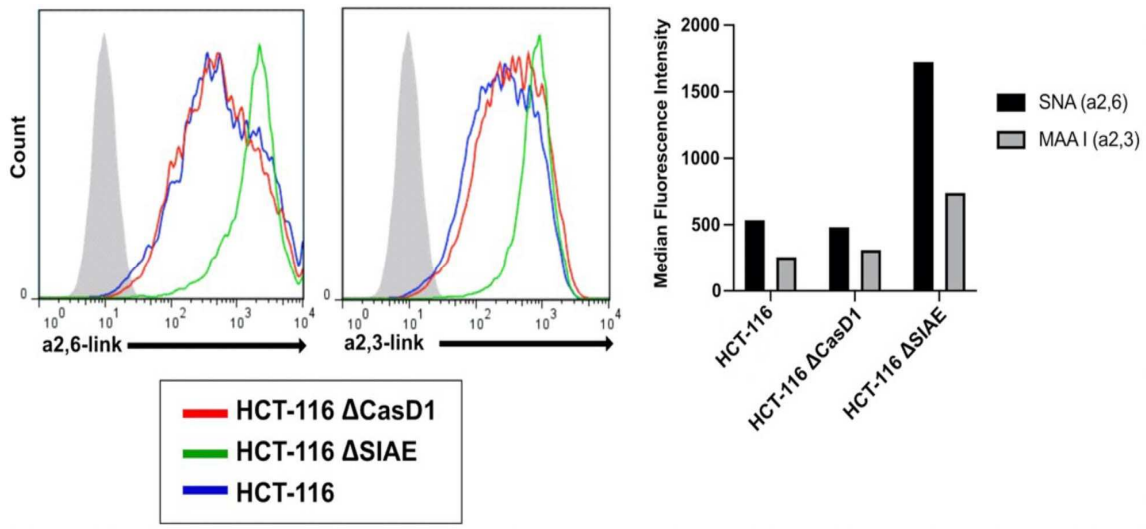


Figure1.13 The quantity of α2–3 and α2–6-linked Sia in HCT 116 ΔSIAE and ΔCASD1 cell lines. Using standard plant lectins the quantity of α2–3 and α2–6-linked Sia were measured and found to be substantially higher in HCT 116 ΔSIAE.

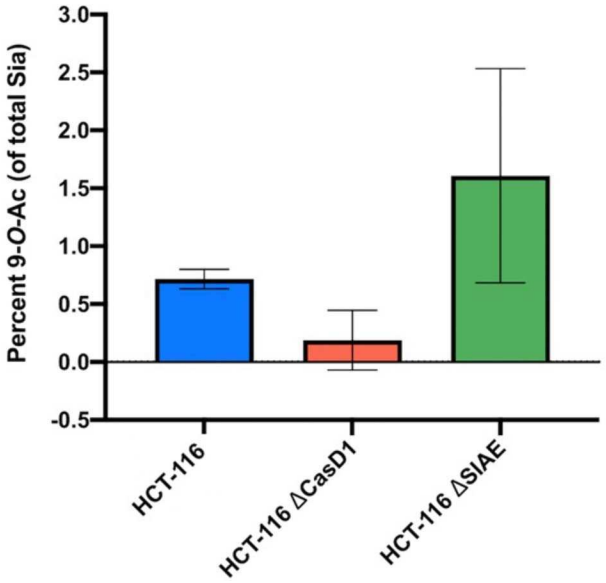


Figure 1.14 Relative amount of the 9-O-Acetyl functional group present on HCT116 cell lines. Higher levels of 9-O-Ac was present on ΔSIAE opposed to the ΔCASD1 cell line. Error bars represent standard deviation of triplicate samples.

1.4.5 Effects Of 9-O-Acetylation On NK Mediated Cytotoxicity

Having identified the modulatory effects of 9-O-acetylation on Siglec binding, we next sought to understand how the modulation of 9- O-acetylation effects NK-mediated cytotoxicity. Cancers use atypical structural forms of sialic acid to suppress NK cell mediated cytotoxicity (Butor *et al*., 1993; Beckhardt *et al*., 1995; Sardari *et al*., 2012; Parameswaran *et al*., 2013; Buchner and Muschen 2014; Laubli *et al*., 2014).

Consequently, cancer cells may decrease their susceptibility to NK-mediated cytotoxicity by overproducing Siglecs 7 and 9 ligands, resulting in immune evasion (Hudak *et al*., 2014; Jandus *et al*., 2014). Specifically, 9-O-aceylated Sias found on Siglec ligands have been shown to prevent inhibitory signals from being propagated on immune cell. It was previously established that 9-O-acetylation of Sias can mask Sia-containing ligands from being recognized by Siglec receptors, resulting in the altered immune response (Sjoberg *et al*., 1994). With our prior data in hand concerning Siglec binding, we sought to quantify the modulatory effects 9-O-acetylation has on immune cell-mediated cytotoxicity, revealing how removal of O-acetylation promotes immune evasion via the Sia-Siglec immune evasion pathway. Control and ⊿SIAE cell lines were incubated with NK-92 cells, at tumor:effector ratios that were titrated for each cell lines. The inability to remove the acetyl functional group, and thus engage Siglecs 7 and 9 (Figs 1.6 and 1.6), resulted in an increase in NK mediated cytotoxicity in ⊿SIAE cell lines (Figs 1.15 and 1.16). In line with prior research (Xiao *et al*., 2016), similar cytotoxicity occurred when control cell lines were pre-treated with neuraminidase (Fig 1.17). Compared with the noncancerous kidney cell lines, both cancer lines HCT 116 (Fig 1.15A-D) and A549 (Fig 1.15E-H) cells were more susceptible to NK mediated cytotoxicity with the ⊿SIAE cell lines. HCT116 (D) and A549 (H), showed an increase in susceptibility compared with the control (Figs 1.15B and K and 1.16). The noncancerous kidney cells were not susceptible to NK mediated cytotoxicity (Fig 1.15J and L)

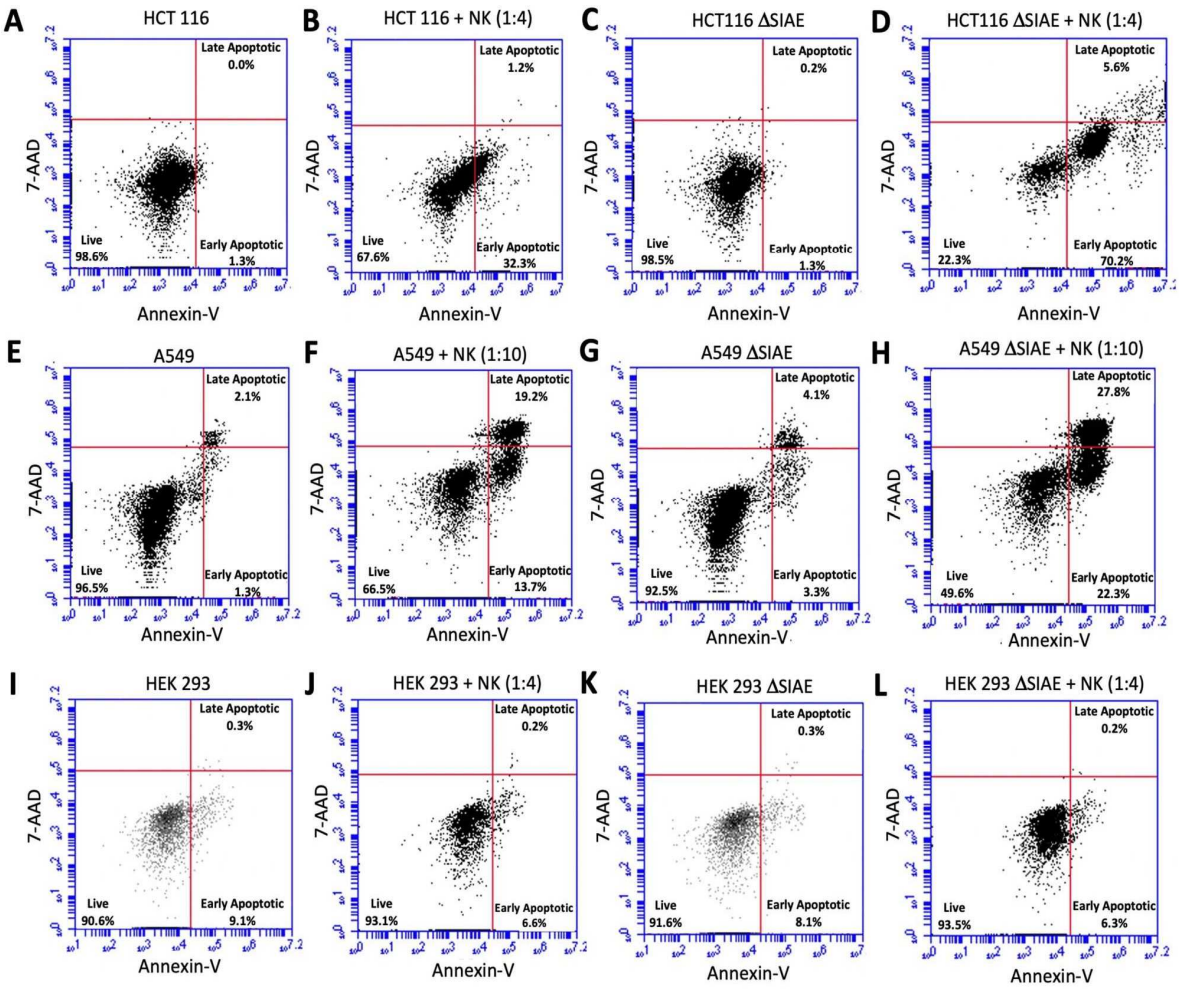


Figure 1.15 NK mediated cytotoxicity assay. Control, ΔSIAE and noncancerous cells were labeled with CellTracker Green CFMDA dye. Activated NK-92 cells were then added to the labeled tumor and kidney cells at ratios (Target: Effector) of 1:4 for HCT 116 (A–D), a 1:10 for A549 (E–H) and a 1:4 for HEK 293 (I–L). Incubation occurred for 4 h at 37◦C. After gating on CFMDA+ cells, cell viability was assessed by analyzing APC Annexin V and 7-AAD. In the absence of SIAE cancer cells HCT116 (D) and A549 (H) were more susceptible to NK mediated cytotoxicity compared with the controls (B) and (K), respectively. Noncancerous kidney cell lines were not susceptible to NK mediated cytotoxicity (J and L).

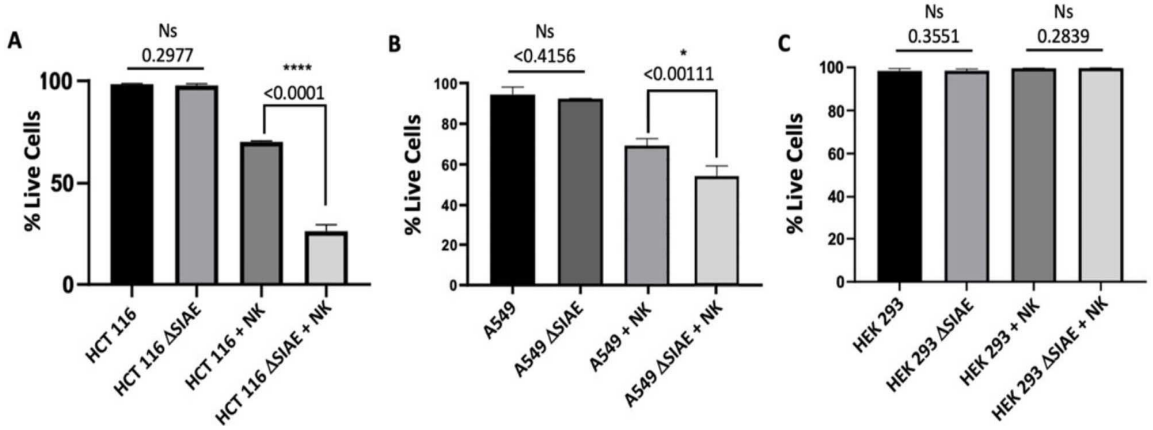


*Figure 1.16 Cytotoxic analysis of NK-92 cells against different SIAE expressing cancer cells. Cytotoxicity assays were performed with NK-92 cells and target cells at a 4:1 ratio for HCT 116 (**A**), 10:1 ratio for A549 (**B**) and a 4:1 ratio for HEK 293 (**C**). Error bars represent standard deviation of triplicate samples.*

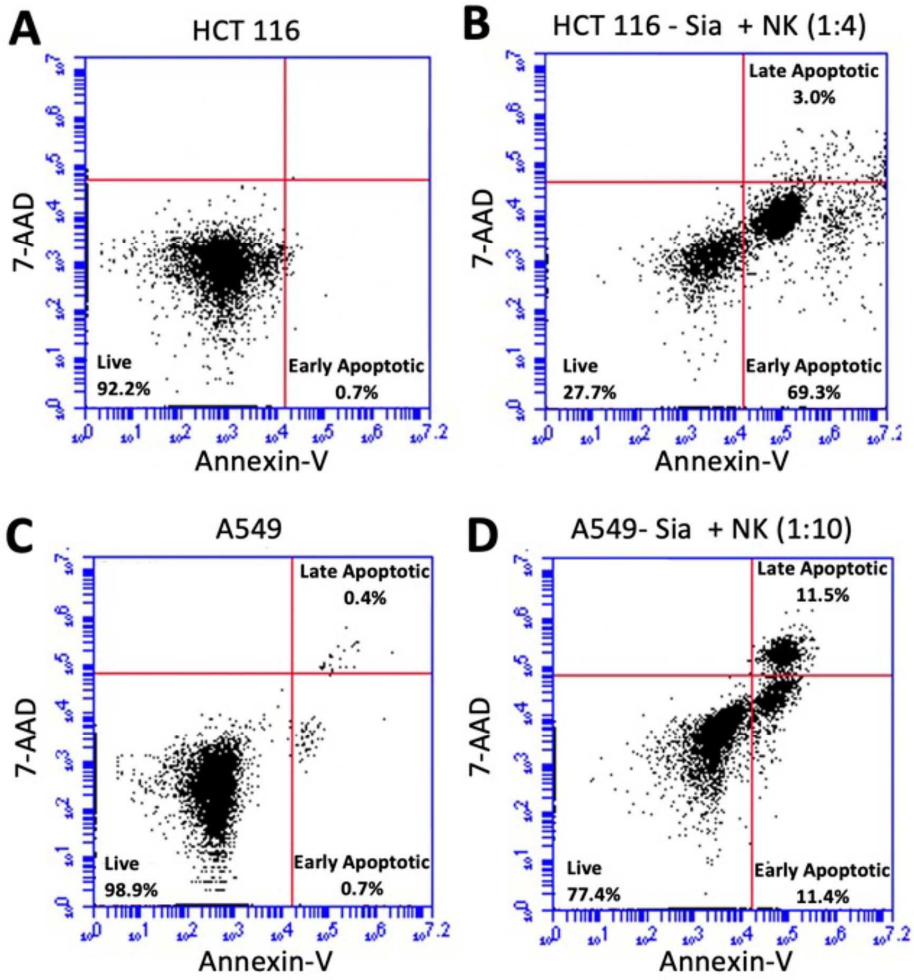


Figure 1.17 NK mediated cytotoxicity assay post neuraminidase treatment. Control, OSIAE HCT116 and A549 cells were labeled with CellTracker Green CFMDA dye and treated with neuraminidase. Activated NK-92 cells were then added to the tumor cells without or with neuraminidase at ratios (Target: Effector) of 1:4 for HCT116 (A–B) and 1:10 for A549 (C–D). Incubation occurred for 4 h at 37◦C. After gating on CFMDA+ cells, cell viability was assessed by analyzing APC Annexin V and7-AAD. In the absence of sialic acid cancer cells HCT116 (B) and A549 (D) were more susceptible to NK mediated cytotoxicity compared with the controls (A) and (C), respectively.

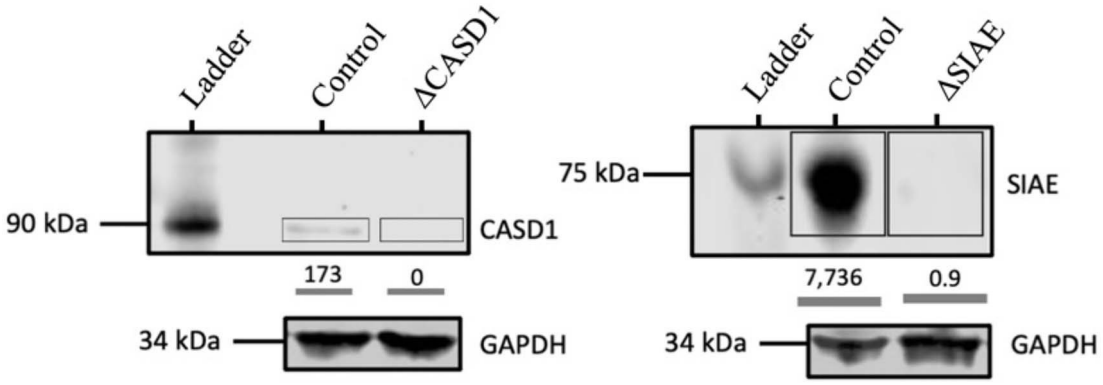


Figure 1.18 Western blot analysis of cell extracts (45 µg) from HCT 116, HCT 116 OCASD1 and HCT 116 OSIAE cell lines.

Sia are modified by many different functional groups, including hydroxyls, O-acetyl, O-

methyl, O-sulfate, O-phosphate and/or O- lactyl groups, and are also attached via different linkages, allowing it to communicate information between proteins, pathogens and various cell types—including cancer cells. Novel Sia forms are specifically selected by cancers to alter their communication with receptors on immune cells, and the communication between cancers and immune cells via the Sialic acid-Siglec pathway appears to be particularly susceptible to manipulation (Varki *et al*., 2015). Modulating acetylation levels on sialic acid can result in either hyperactivation of immune cells or inhibition of immune cell mediated cytotoxicity (Shi *et al*., 1996). In addition, the level of Sia acetylation has been shown to be phenotypically associated with certain cancers, although, depending on the cancer, the functional group may be up or down regulated (Pearce and Laubli 2016). For example, half of Sias found on colonic mucins in healthy tissues were acetylated (Shen *et al*., 2004), and de- acetylation of sialic acids was identified in all stages of colon cancer, and was seen to be an early alteration accompanying the adenocarcinoma sequence in cultured cells (Corfield *et al*., 1999; Shen *et al*., 2004). Reduced acetylation of sialic acids also occurs on sialyl Lewis X motifs, a tumor-associated antigen, and that is a primary alteration related to progression and metastasis of colorectal cancer (Mann *et al*., 2012; Pretzsch *et al*., 2019). We sought to understand how modulating acetylation would affect cancer associated Siglec binding (Siglecs 7, 9, 10 and 11), as well as to understand their ability to thwart immune mediated cytotoxicity via NK cells utilizing this functional group. Our data suggests that de-acetylated Sias increase Siglec receptor binding and engagement in immune cell mediated cytotoxicity, via the Sia-Siglec pathway. After knocking out CASD1 and employing esterases, the removal of acetylation from the C6 tail of Sias resulted in an increase in Siglecs binding to colon (HCT 116) and lung (A549) cancer cell

lines, compared with a noncancerous kidney cell line (HEK 293). In addition, NK mediated cytotoxicity was increased in the presence of 9-O and 7,9-O-acetylated C6 tail but was decreased when the acetyl groups were removed.

Initial experiments explored how acetylation affected Siglec binding utilizing commercially available reagents, in addition to which acetylated positions contributed to binding (C7 and C9). Removal of SIAE and CASD1 via CRISPR Cas9 gene editing directly affected Siglec binding. In the absence of SIAE, 9-O-acetyls were present and Siglec binding was either blocked or significantly reduced in Siglecs 7, 9, 10 and 11. Siglec binding was greatly increased, however, in cell lines in which CASD1 had been removed. As acetylation can occur at C7 and C9 (to a lesser extent C8) of the glycerol side chain on Sia, we selectively removed either the 9-O or 7,9-O-acetyl using specific virus-derived esterases, resulted in an increase of Siglec binding in both instances in colon cancer cell line HCT 116, indicating that both positions can modulate Siglec receptor binding.

Building off the Siglec binding assays, we explored the modulatory effects 9-O-aceyaltion had on NK mediated cytotoxicity. NK cells are a crucial component in the immune system's defense against cancers, and the balance of inhibitory (via Siglec 7 and 9) and activating signals at the immune synapse determines an NK cell's ability to differentiate between healthy tissues and cancerous cells (Xiao *et al*., 2016). Yet, in the tumor microenvironment cancer cells evade NK mediated destruction by aberrantly expressed glycoproteins and glycolipids, which aid in the recruitment of Siglec 7 to the NK cell synapse, resulting in inhibitory signaling through its ITIM domain (Hudak *et al*., 2014). It had yet to be determined how functional group alterations of Sias specifically contribute to an altered Sia-Siglec immune evasion pathway. Prior research demonstrated

that Siglec 7 and 9 preferentially binds to deacetylated Sias and that binding is lost in the presence of 9-O-aceylation (Sjoberg *et al*., 1994; Nicoll *et al*., 2003; Avril *et al*., 2006). Siglec 9, which is generally over expressed in cancers, specifically functions to inhibit NK-cell-mediated antitumor response by acting as an anti-adhesive molecule, preventing the target cell and NK cell from forming an immunological synapse. Our NK mediated cytotoxicity data conducted on ⊿SIAE cell lines revealed that increased acetylation of the Sia resulted in increased cytotoxicity, suggesting that the acetyl group interferes with the engagement of the Siglec inhibitory receptors.

Sialic acid linkages normally exist in three main configurations: α2,3, α2,6 and α2,8, all catalyzed by a group of sialyltransferases (Lu and Gu 2015). Our investigations revealed a link between the expression of SIAE and the Sia α2,3 and α2,6 linkages. Changes in Sia linkages have been noted in cancers with α2–6 linked Sia becoming particularly prominent. Increased α2,6 sialylation (catalyzed by β- galactoside α2,6 sialyltranferase) has been observed in many cancers (Lu and Gu 2015) and increases in α2–6 Sia enhances tumor progression and poor prognosis in later stages of cancers. Alterations of α2– 6 Sia expression were first described in colon cancer (Dall'Olio *et al*., 1989; Sata *et al*., 1991; Vierbuchen *et al*., 1995), but later confirmed in several others, including, breast (Recchi *et al*., 1998), liver (Dall'Olio *et al*., 2004; Jin *et al*., 2004), cervix, choriocarcinomas, acute myeloid leukemias and malignancies of the brain. A significant increase of α2,6 sialylated was also associated with increased metastatic tumor growth of colon cancers (Vierbuchen *et al*., 1995). Interestingly, in the HCT 116 ⊿SIAE cell line there was a dramatic increase in α2–6- linked Sias and a small increase in α2–3-linked Sias. It is unclear how the SIAE gene would influence expression of those specific linkages. Several mechanisms have been proposed

for the regulation α2–6- linked Sia via ST6Gal I (Lu and Gu 2015). In ST6Gal I knockout mice, highly selective loss of 9-O-acetylation of Sias has been observed (Martin *et al*., 2002). Our results suggest that 9-O-acetylation is associated with the generation of α2–6-linked Sias via ST6Gal I, including a modulatory role by SIAE.

Although Siglecs have sialoside preferences, modulation of 9-O- and 7,9-O acetylation appears to influence Siglec binding to a greater extent than the glycosidic linkage—although Siglec 7, 9 and 10 are capable of binding to α2–6-linked Sias (Crocker *et al*., 2007), in the α2–6-linked Sia overexpressing HCT 116 ΔSIAE cell line, Siglec binding was lost in the presence of acetylation.

SNA and MAA binding also indicates that SIAE appears to play a role in regulating the level of Sia present in the cell. The relative amounts of the 9-O-acetylation were measured from total Sia expressed on HCT116 cell lines. Increased total Sia levels were found in the HCT 116 ΔSIAE, compared with ΔCASD1, cell lines indicating that the Sia are not being degraded at the same rate. Activated Sias such as CMP-Neu5Ac are imported into the Golgi compartment where they are acetylated via CASD1, prior to being transported to the cell's surface (Barnard *et al*., 2019). Sia-glycoproteins may also be recycled from the cell surface and during the trafficking be de- acetylated by the Lse and Cse variants of SIAE in the lysosome and cytosol respectively. It is possible that without the ability to de-acetylate the enzyme sialate pyruvate-lyases, also known as Sialate Aldolase, is unable to reversibly catalyze the cleavage of free Neu5Ac to form pyruvate and ManNAc. Generally, ManNAc would then reenter the Sia biosynthetic pathway after further phosphorylation or be metabolized (Bulai *et al*., 2002, 2003). The O- acetylated forms of Sia have been reported to be poor substrates for CMP-Neu5Ac synthase, yet levels of 9-O-aceylated Sias

were still found on the surface of the cell. This could be explained by the presence of the Cse variant of SIAE, assuring a higher efficiency in the recycling of Sias in the cytosol. Although a single Sia 9-O- acetylesterase gene encodes both variants, there may be different levels of expression of the variants in different cells. (Takematsu *et al*., 1999) The continued presence of acetylated Sias on the surface of the cell can also be explained by the presence of CASD1 in the cell which would continue to generate CMP-Neu5,9Ac2 for use by sialyltransferases (Baumann *et al*., 2015).

Collectively, these studies highlight the fundamental modulatory role variation that acetylation plays on Sia towards various aspects of cancer biology.

1.5 Conclusion

The presence of acetyl functional groups on position 9-O or 7,9-O of terminal Sia of glycoproteins and glycolipids has been shown to preclude the recruitment of Sia-binding inhibitory receptors, Siglecs. This was seen to reduce the inhibitory signals sent from tumors to NK cells and thus makes tumor cells more susceptible to NK-mediated cytotoxicity. This research has provided evidence of the possibility of improving immune response against cancers by simply targeting and inhibiting the expression of the SIAE gene or its protein product. This avenue could be further exploited in developing effective immunotherapies that can be used singly or in combination with other treatments against cancer.

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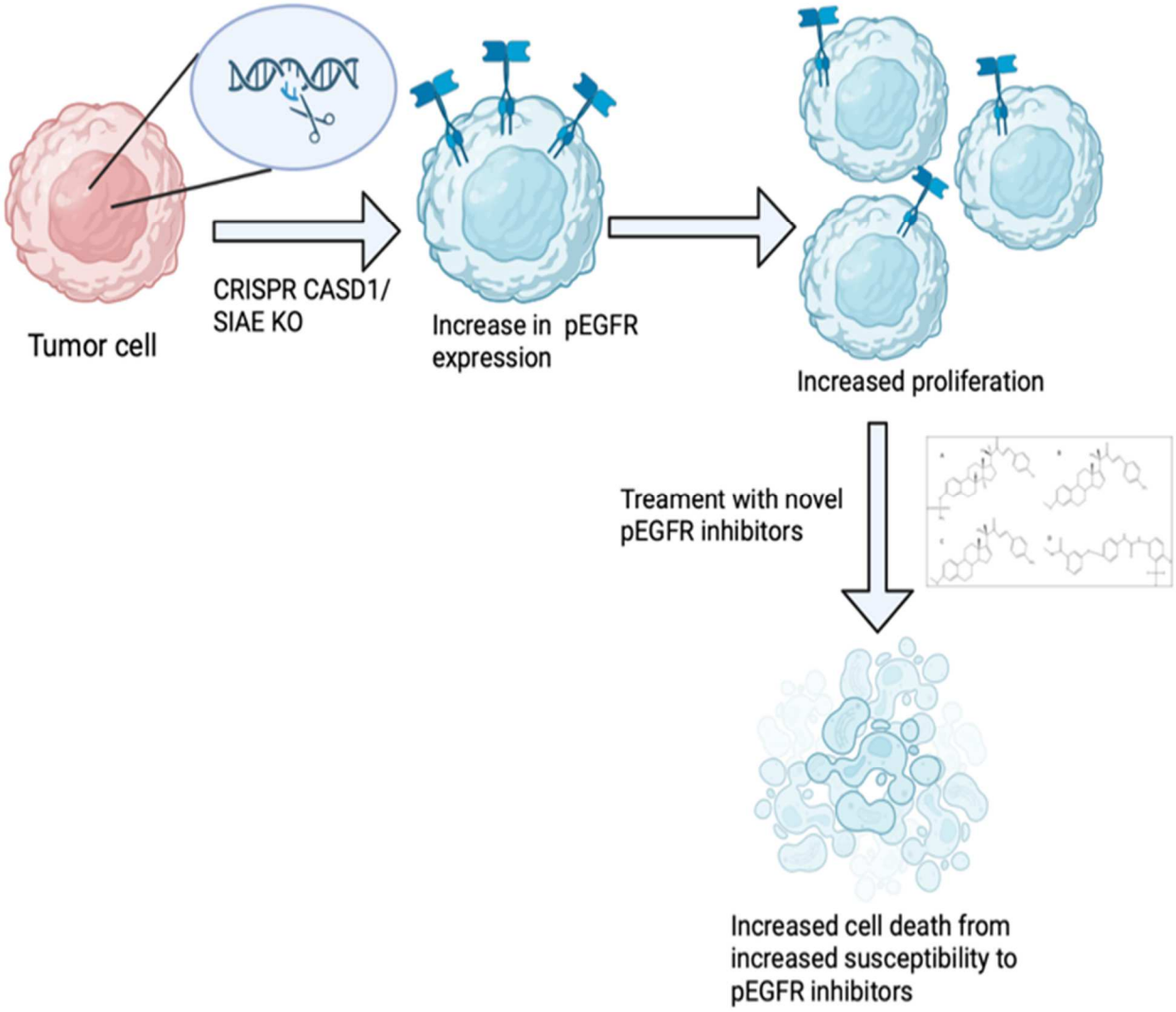
Chapter 2

Aberrant CASD1 And SIAE Expression Renders Lung And Colon Cancers Susceptible To Epidermal Growth Factor Receptor Inhibitors



2.1 Abstract

Cancers utilize simple sugar residues to improve their ability to survive and metastasize. Overexpression of sialic acids (Sia), a 9 carbon sugar found in the cell's glycocalyx has been linked to poor disease prognosis and treatment response making cancer one of the major causes of mortalities across the globe. This has been identified as a hallmark of cancer and its progression. Sialic acid can be highly functionalized, but we became interested in acetylated Sias because aberrant Sia acetylation has been linked to loss of drug sensitivity and an escape from apoptosis in tumors. This functional group is modulated by Sialate O- acetylesterase (encoded by the gene SIAE) which removes acetyl groups and Sialate O-acetyltransferase (encoded by CASD1) which acetylates the 7 and 9-O positions of Sia. Aberrant expression levels of CASD1 and SIAE have been linked to a dysregulation of apoptosis, rendering certain tumor cells highly insensitive to certain drugs that are meant to stimulate apoptosis. In this study we investigated the effect of CASD1 and SIAE dysregulation in HCT116 and A549 cells on their sensitivity towards a known drug and three novel EGFR inhibitors. on Our findings show that targeting SIAE and CASD1 in chemotherapy renders it more susceptible to drugs that target and inhibit tumor cell proliferation via EGFR signaling. Chemically synthesized novel hybrid analogs (MMA 294, 320 and 321) and Sorafenib exhibited an apoptotic mode of cytotoxicity on colon and lung cancer cells when analyzed with the Annexin V/7-AAD assay. The effect of these drugs on the cell cycle of HCT116 and A549 cells was also assessed using DNA staining with propidium iodide. MMA 294 and sorafenib elicited a G_0/G_1 cell cycle arrest in A549 WT and ΔCASD1 cells but a G_2/M in A549 SIAE. MMA 321 also showed a switch in cell cycle arrest from G_0/G_1 in WT and HCT116 ΔCASD1 to a G_2/M in HCT116

SIAE cells. Further screening of activated EGFR by immunofluorescence revealed an overexpression of pEGFR in the CASD1 and SIAE knockout cells suggesting an increased activation of this growth receptor rendering the knockout cells highly proliferative but extremely sensitive to the novel hybrid analogs of estrone origin.

Keywords: Sialate-O-acetyltransferase, sialiate-O-acetylesterase, propidium iodide, epidermal growth factor receptor (EGFR).

2.2 Introduction

Sialic acids, the terminal sugar residues on sialoglycans are important sugar residues that play key roles in intercellular communication and migration. Surprisingly, their functionalization offers them with various unique capabilities, most of which are still unraveling. The acetylation or deacetylation of Sia controlled by CASD1 and SIAE has been proven to affect immune cells' activity, and growth of tumors. (Sjoberg *et al*. 1994; Grabenstein *et al*., 2021). More specifically, when these Sia functionalizing enzymes are aberrantly expressed, tumors tend to grow rapidly and undergo apoptosis much slower than expected (Mather *et al*., 2019). The response of such tumors to chemotherapy is thwarted in such scenarios, often rendering chemotherapy a less efficient treatment option to alleviate the disease. Targeted therapies could be a better treatment regimen when conventional chemotherapeutics do not work effectively in such situations. Targeted therapy in cancer involves the use of small drugs or molecules to specifically identify cancer cells based on their unique phenotypes that sets them apart from normal cells (Peters, 2019). This approach tends to attack cancer cells more specifically with very little

harm to normal cells, thus leading to fewer side effects to patients when targeted therapy regimens are given. Brodsky's review on monoclonal antibodies as magic bullets was one of the few papers that discussed the possibility of expanding a targeted therapeutic approach into the fight against cancer (Brodsky, 1988). In 1995, the first ever kinase inhibitor called Fasudil, a Rho kinase inhibitor was approved in Japan for the treatment of cerebral vasospasm (Shibuya *et al*., 1993). Though the first kinase inhibitor approved was not an anticancer agent, subsequent years saw a huge interest in kinase inhibitors as treatment towards cancer. As such, in 1999, sirolimus, an allosteric kinase inhibitor was approved for the treatment of organ rejection in patients undergoing kidney transplants. Subsequently imatinib became the first approved small molecule kinase drug for fighting cancer (Buchdunger *et al*., 2001, Kahan, 1997). Trastuzumab was also approved in 1998 as the first monoclonal antibody drug that targets HER-2 and used in treating HER-2 positive cancers.

Targeted therapies are usually small molecules or monoclonal antibodies that are designed to deliver substances to the immune system, thus improving their effectiveness or they could also be drugs that are toxic and delivered directly to cancer cells to stop proliferation, metastasis or induce cytotoxicity. Small molecules have a limited molecular weight and their small size gives them the ability to penetrate into cells to inhibit specific enzymes or molecules that play key roles in signaling pathways (Xie *et al*., 2020). Monoclonal antibodies on the other hand targets cell surface receptors and regulate their downstream signaling pathways in order to alleviate a disease condition.

2.2.1 Receptor Tyrosine Kinases As Effective Molecular Targets

Some key pathways that have been successfully targeted to alleviate cancer are the receptor tyrosine kinase signaling pathways. These are pathways that involve kinases and phosphatases which catalyze the sequential phosphorylation and dephosphorylation of molecules in the cell. Kinases in general form about 5% of all the protein coding genes in the entire human genome. However, they play crucial roles in the survival of the cell, transferring gamma phosphate groups from (adenosine triphosphate) ATP to a wide range of hydroxyl containing molecules. Their diversity in terms of roles allows them to control cell division, proliferation and even metabolism of nutrients in cells. Any dysregulation of kinases leads to aberrant phosphorylation of substrates in signaling pathways and this has been linked to diseases like cancer, diabetes, cardiovascular diseases and inflammatory disorders (Cohen, 2001).

Kinases are grouped and named depending on the substrates that receives the phosphate groups. For instance, serine/threonine kinases transfer phosphate groups from ATP to serine or threonine amino acids of the receiving protein while tyrosine kinases perform the same task but to tyrosine residues. Other well-known kinases are nucleotide kinases, lipid kinases, sugar kinases and dual-specificity kinases (Fabbro *et al*., 2015). One main kinase of interest is the epidermal growth factor receptor which is a tyrosine kinase that controls several downstream regulatory pathways.

The Epidermal growth factor receptor (EGFR) is the first member of the ErbB (erythroblastosis oncogene B)/HER (human epidermal receptor) growth factor receptor family of proteins. EGFR is a transmembrane glycoprotein that is made up of an

extracellular domain, a transmembrane domain, an intracellular tyrosine kinase domain and a C-terminal domain that plays key regulatory roles.

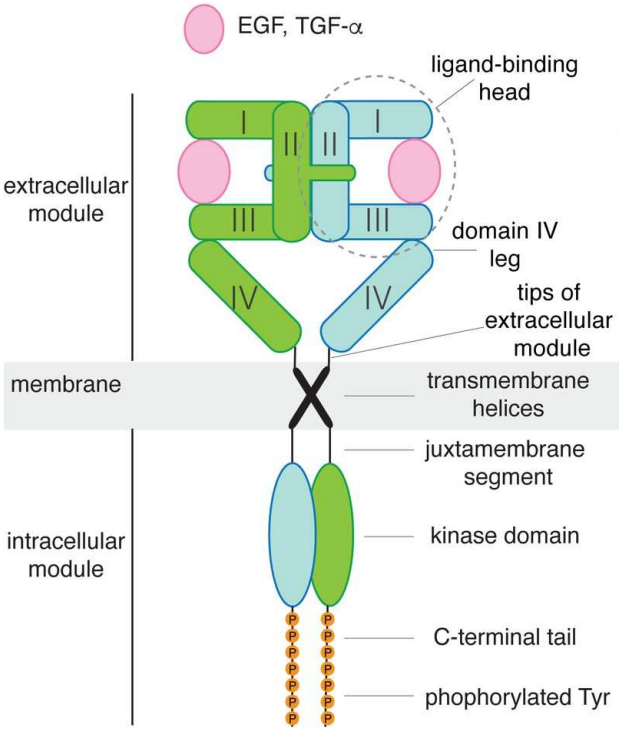


Figure 2.1 Detailed structure of the epidermal growth factor receptor

The extracellular domain is post translationally modified by the attachment of gycosyl units to Asn residues which regulate the receptor (Whitson *et al*., 2005). The extracellular domain is heavily N-glycosylated with the sugar residues making up 40kDa of the 180kDa mass of the protein (Soderquist and Carpenter, 1984). In all, 11 canonical N-glycosylation sites have been mapped on the ectodomain of the EGFR with each site specified by the sequence NXS/T, where X can be any amino acid aside proline (Ullrich *et al*., 1984). Glycosylation is key to the proper functioning of the receptor. Specifically, when EGFRs are synthesized in the presence of glycan transferase inhibitors, the resulting non-glycosylated EGFRs lose the ability to bind its cognate ligands and surprisingly lose the intracellular kinase activity (Cummings *et al*., 1985).

Whitson et al (2005) performed a site directed mutagenesis of a glycan site to investigate the effect of glycosylation at this site on receptor function. In their research an Asn to Gln mutation was performed at residue 579 which resulted in loss of glycosylation at this site. Whitson et al observed that cells expressing N^{579} glycosylated EGFR exhibited an IL3 independent survival in the presence of normal growth media with serum. However, those that expressed the N^{579} non-glycosylated EGFR required IL-3 in order to survive, suggesting that the glycan at this position modulated cell-signaling pathways that support survival.

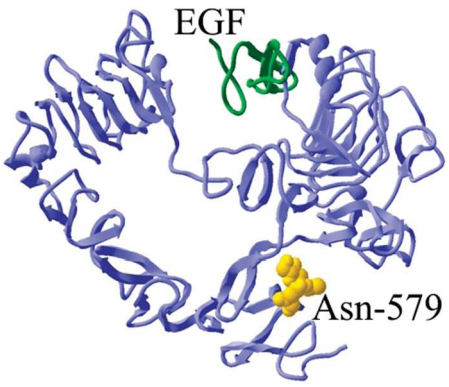


Figure 2.2 Structure of EGF-bound EGFR showing the N579 glycan chain needed for survival of cells

EGFR has about seven different agonists that can bind to its extracellular ligand binding domain and triggers stabilization and dimerization of the receptors and activation of the intracellular kinase domain via a trans autophosphorylation of its tyrosine kinase domain (Dawson *et al*., 2005). The epidermal growth factor (EGF), amphiregulin (AR), betacellulin (BTC) and the transforming growth factor α (TGF-α) are among the most common agonist of the EGFR. Activation of EGFR can also occur when its ligands bind to already preformed dimers of the receptor where each monomer is in an extended state (Azimzadeh Irani *et al*., 2017).

Trans autophosphorylation of tyrosine residues leads to the recruitment of Src Homology 2 (SH2) and/or phosphotyrosine binding (PTB) containing signaling molecules that further leads to the activation of the multiple intracellular downstream signaling pathways (Cadena *et al*., 1994). At least 3 of these pathways namely the RAS/RAF/MEK/ERK pathway, JAK/STAT3 pathway and the PI3K/AKT pathway directly control cell growth, differentiation and migration (Wang, 2017).

The RAS/RAF/MEK/ERK pathway often referred to as the mitogen-activated protein kinases (MAPK) pathway is one of the well-studied and highly conserved cascade mechanisms in eukaryotes. It generally involves the relay of upstream ligand-bound activation signals of the EGFR to downstream protein kinases that ultimately activate genes in the nucleus that are involved in cell proliferation, differentiation and survival (Wang, 2017). The cascade begins when phosphotyrosine residues in the intracellular domain of activated EGFR recruits SH2 containing Grb2 protein which further recruits another protein called the Son of sevenless (Sos). As soon as Sos gets anchored to the receptor via Grb2, it is brought to close proximity with the Ras proteins, most of which is found tethered to the inner plasma membrane through its C-terminal hydrophobic tail. Since Sos is a guanine exchange factor, its close proximity to Ras equips it with the ability to interact with the tethered Ras molecules and induce the release of GDP and the binding of GTP instead to Ras, thus activating Ras. GTP-bound Ras has a 3-fold increased affinity for Raf and binds to it via it's effector loop (Chang and Karin, 2001). The previously cytosol bound Raf, then gets anchored to the plasma membrane via its association with membrane-bound activated Ras. There are several other scaffolding proteins that hold this association together, ensuring that activated Raf proceeds to phosphorylate the next kinase in the

cascade known as the mitogen-activated protein/extracellular signal-regulated kinase (MEK) (Brown and Sacks, 2009). MEK has the ability to phosphorylate both serine/threonine residues and tyrosine residues on subsequent kinases as such is referred to as a dual-specificity kinase. Activated MEK phosphorylates two other kinases, the extracellular signal-regulated kinase 1 (Erk1) and Erk2.

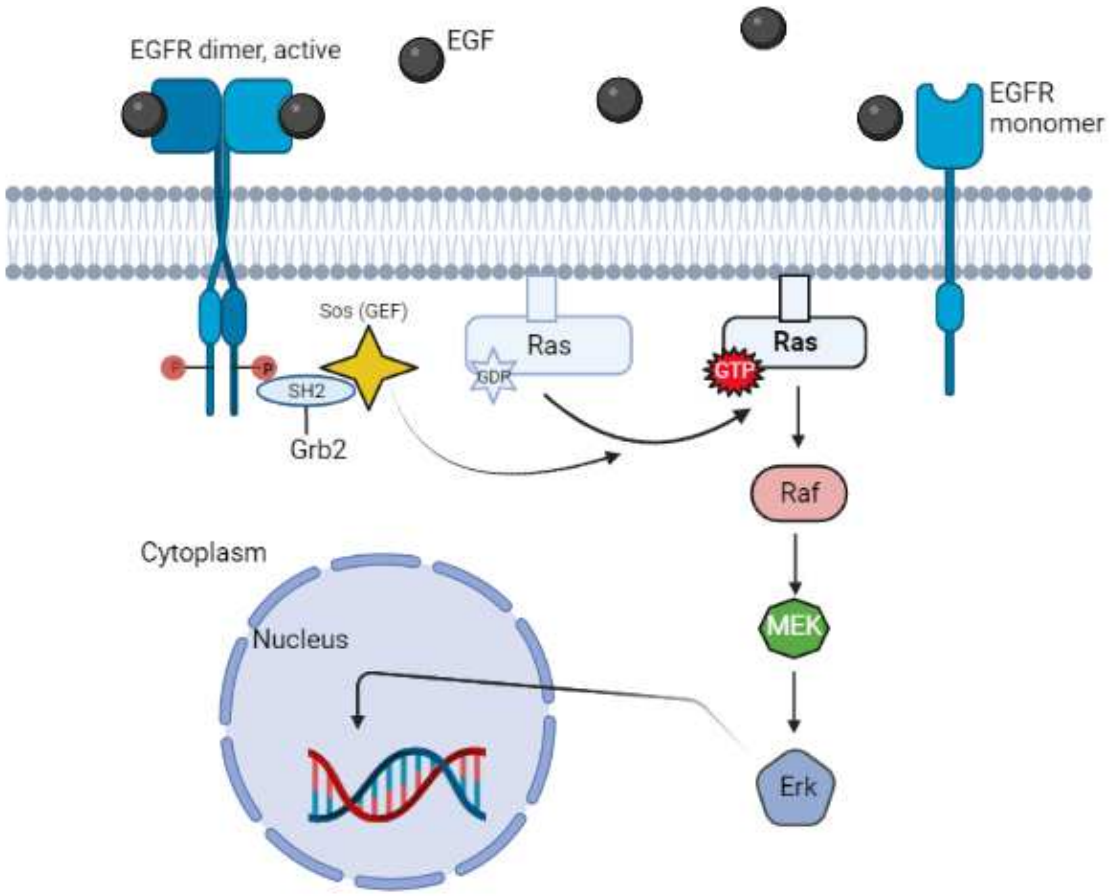


Figure 2.3 The MAPK Signaling Pathway showing the binding of EGF to EGFR and subsequent downstream activation of the various effector proteins

Once activated, Erk, which is at the bottom of this cascade phosphorylates both cytoplasmic and nuclear substrates, the latter being transcription factors such as Elk-1 and Ets that activates genes in the nucleus (Chang and Karin, 2001). Ets is a transcription factor

that specifically stimulates the expression of important growth-regulating genes such as genes encoding cyclin D1, heparin-binding EGF and Fos. Erk also phosphorylates other downstream kinases that phosphorylates two chromatin-associated proteins (HMG-14 and histone H3), a unique modification that makes the chromatin much more hospitable to transcription (MacCorkle and Tan, 2005).

Several studies have reported that EGFR is aberrantly expressed in different cancer types and the expression levels dictates the prognosis of most of these cancers. Lung, prostate, colorectal, breast, bladder and pancreatic cancers are some examples of cancers that overexpresses EGFR. It has been reported that 60% of all NSCLCs and 25 to 77% of colorectal cancer patients have an increased level of expression of EGFR (Rotow and Bivona, 2017).

EGFR can be targeted in cancers to alleviate and treat the disease. This can be done via an anti-EGFR monoclonal antibody or tyrosine kinase inhibitors that specifically inhibit intracellular kinases. In 1995, cetuximab was named as the first monoclonal antibody that targets EGFR and induces receptor internalization and degradation when it binds to EGFR (Prewett *et al*., 1996). In February 2004, cetuximab was approved by the US Food and Drug Administration (FDA) for the treatment of irinotecan refractory or intolerant metastatic colorectal cancer (Cunningham *et al*., 2004). In 2011, it was given further approval for use in treating late-stage head and neck cancer.

This study aims at investigating the impact of novel EGFR inhibitors on colon and lung cancer cells that have an aberrant expression of CASD1 and SIAE. The novel hybrid analogs of estrone origin have been proven to be EGFR inhibitors by Acheampong (2019) and will their effectiveness against CASD1 and SIAE gene disrupted lung and colon cancer

will assessed. This research specifically investigates MMA 294, MMA 320 and MMA 321 which showed the highest cytotoxic activity during the preliminary screening.

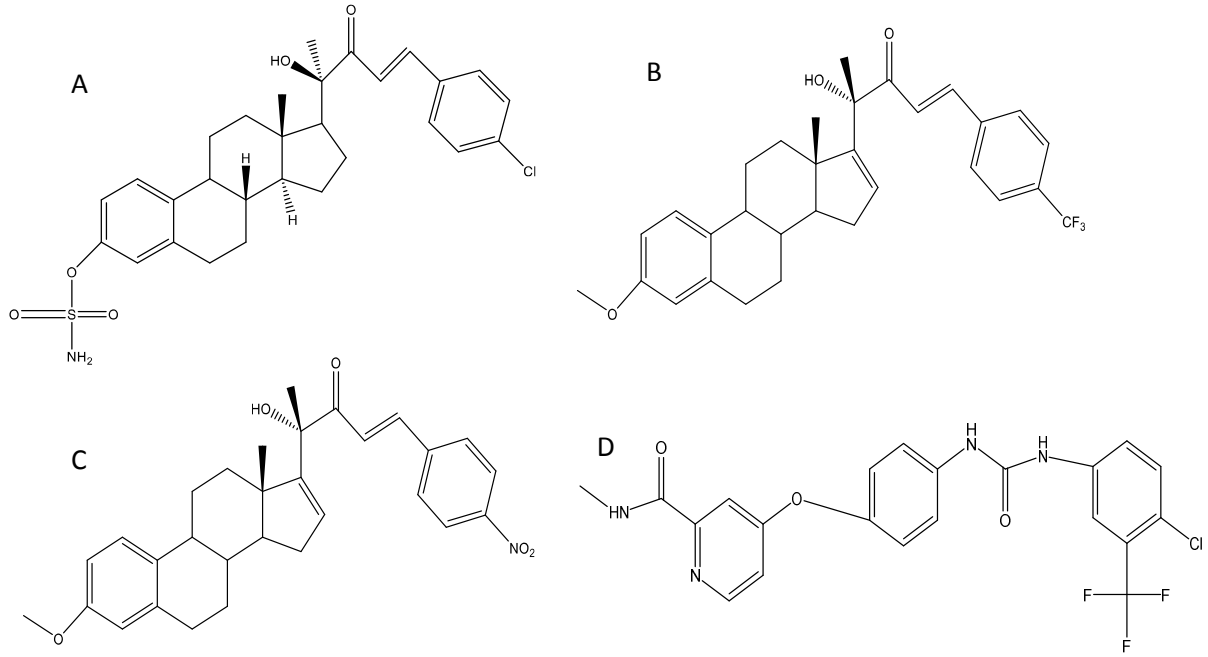


Figure 2.4 Structural formula of novel hybrid analogs of estrone origin (A) MMA 294 (B) MMA 320 (C) MMA 321 and (D) Sorafenib

Mather et al. (2019) reported that, a typical dysregulation of these Sia-modifying enzymes in malignant brain tumor cells tends to protect these brain tumor cells from apoptosis. Medulloblastoma brain tumor cells have evolved a unique pathway to protect itself and ensure tumor cell survival by simply dysregulating the expression pattern of its cognate CASD1 and SIAE. Because the ganglioside GD-3 (which happens to be the product of the SIAE reaction) is proapoptotic, it's expression levels is downregulated while GD-3A (the product of the CASD1 reaction) is upregulated since it is antiapoptotic. This makes medulloblastoma brain tumor cells highly proliferative and offers them a much

longer lifespan. The disruption of this dysregulation by simply upregulating SIAE and downregulating CASD1 surprisingly caused an increase in the tumor's sensitivity to etoposide (Mather *et al*., 2019). In other words, by targeting and disrupting CASD1 and SIAE expression Mather et al. made medulloblastoma much more vulnerable to treatment with etoposide. This indicated the possibility of drug insensitivity of certain tumors especially when CASD1 and SIAE levels are altered and thus by altering CASD1 and SIAE levels we can improve drug treatment of those cancers. This research sought to investigate how such dysregulation in expression of SIAE and CASD1 would affect a targeted therapy regimen that focuses on the phosphorylated epidermal growth factor receptor (EGFR). The EGFR is a well-known molecular target in targeted therapy and one of the few targets whose activity is greatly influenced by its level of sialylation. For instance, hyper-sialylation of its N-linked glycans has been shown to attenuate dimerization of its extracellular domain consequently downregulating its autophosphorylation sites in lung cancer (Yen *et al*., 2015). Ling et al. (2009) also observed an increase in NSCLC susceptible to erlotinib if EGFR's N-glycosylation is inhibited with tunicamycin. We sought to understand the modulatory effects of 7 and 9-O-acetylated Sias on the sensitivity of A549 and HCT116 cells' cognate EGFR to its inhibitors without altering the sialylation levels of this receptor.

The study aimed to specifically evaluate the effect of acetyl functional group alterations on the viability of tumor cells (HCT116 and A549) treated with a known receptor tyrosine kinase inhibitor (sorafenib) and novel hybrid analogs of estrone origin.

2.3 Materials And Methods

2.3.1 Chemicals And Reagents

Annexin V binding buffer 1x, APC Annexin V, 7-Amino-Actinomycin D (7-AAD) and PI/RNase Staining buffer were obtained from BD Biosciences (San Jose, CA). The tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M-6494 was purchased from Thermo Fisher while the phosphorylated EGFR monoclonal IgG1 mouse antibody (15A2:sc-81488) obtained from Santa Cruz Biotechnology, (Dallas, TX). The secondary Goat Anti-mouse IRDye secondary antibody (LiCor) was purchased from LI-COR Biotechnologies (Lincoln, NE) and the Vectashield Antifade Mounting medium with DAPI (H-1200-10) was purchased from Vector Laboratories (Burlingame, CA).

2.3.2 Cell Lines And Cell Culture

A549 were grown in Dulbecco modified Eagle medium (DMEM Corning) with 10% fetal bovine serum (Corning) and 1% pen/strep (Cytiva Hyclone). HCT 116 cells were grown in RPMI 1640 medium (Corning) with 10% fetal bovine serum and 1% pen/strep. All cell lines were originally purchased from American Type Culture Collection. Concerning the passaging of cells, Cell Dissociation Buffer (Gibco,Waltham, MA) was used exclusively. In the generation of CRISPRi-mediated knockout of CASD1 and SIAE knockout HCT116 and A549 cell lines, the CASD1 and SIAE knockout HCT 116 and A549 cell lines were obtained from the group of Colin Parrish (Cornell University). CRISPR-Cas9 editing of CASD1 and SIAE in HCT116 and A549 cells was previously published (Barnard *et al*., 2019). In brief, for CASD1 knockout cells, paired Cas9 plasmids

targeting adjacent sites in early exons of CASD1 were transfected using TransIT-X2 (Mirus Bio LLC, Madison, WI). Transfected cells were selected with puromycin and single cell clones screened with PToV-P4 HE-Fc and sequence verified to confirm loss of CASD1 function in both alleles (Langereis *et al*., 2015). SIAE knockout cells were prepared using the same method with single cell clones screened by direct PCR amplification of the Cas9 targeted exons. Full sequencing of each allele and qPCR were performed to confirm deletion of the SIAE gene.

2.3.3 Cell Proliferation Assays

2.3.3.1 MTT Cell Viability Assay

HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Corning, USA) with 10% fetal bovine serum and 1% Penicillin Streptomycin (Cytiva) until a cell confluence of 80% was reached. A549 cells were also cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning, USA) with 10% fetal bovine serum (Gibco) and 1% Penicillin Streptomycin (Pen-Strep) until a confluence of 80% was reached. All cells were harvested from culture flask using enzyme-free cell dissociation buffer (Gibco), counted and seeded in 96 well plate at a density of 10,000 cells/well and incubated overnight for cells to adhere. Chemically synthesized phosphorylated EGFR inhibitors was subsequently added to designated wells to obtain final drug concentrations of 100, 50, 25, 12.5 and 6.25μM. Similar dilutions were prepared for Sorafenib which was used as the positive control for all cells. The treated cells were incubated for 48hrs in 5% CO2 and 37°C. The tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Thermo Fisher, USA) was finally added to the wells and further incubated for 4hrs

in the dark in 5% CO_2 and 37°C. Acidified sodium dodecyl sulfate was added to each well to dissolve the formazan crystals formed and absorbance was measured at 570nm using the Cytation 3 Cell Imaging Plate Reader. The percentage cell viability was determined as follows:

$$\%Cell\ viability = \left(\frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ blank}{Absorbance\ of\ untreated\ cells - Absorbance\ of\ blank}\right) x\ 100$$

2.3.3.2 Mode Of Cytotoxicity-Apoptosis Assay

HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum and 1% Penicillin Streptomycin (Pen-Strep) until a cell confluence of 80% was reached. A549 cells were also cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% Penicillin Streptomycin (Pen-Strep) until a confluence of 80% was reached. All cells were harvested from culture flask using enzyme-free cell dissociation media, counted and seeded in 6 well plate at a density of 100,000 cells/well and incubated overnight for cells to adhere. Chemically synthesized phosphorylated EGFR inhibitors and sorafenib were subsequently added to designated wells to obtain final drug concentration of IC_{50} value obtained from MTT cell viability assay. The treated cells were incubated for 48hrs in 5% CO_2 and 37°C. The supernatant was collected and later pooled together with adhered cells that were detached with 0.5mL of cell dissociation buffer. The cells were washed three times in sterile 1x Phosphate Buffered Saline (Corning, USA) and resuspended in Annexin V binding buffer 1x (BD, USA). APC Annexin V (BD, USA) and 7-AAD (BD, USA) were added to the wells (Martin *et al*., 1995). The plate was further incubated in the dark for 25 minutes and read using the BD Accuri C6 Plus flow cytometer.

2.3.3.3 Cell Cycle Analysis

HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum and 1% Penicillin Streptomycin (Pen-Strep) until a cell confluence of 80% was reached. A549 cells were also cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% Penicillin Streptomycin (Pen-Strep) until a confluence of 80% was reached. All cells were harvested from culture flask using enzyme-free cell dissociation media, counted and seeded in 6 well plate at a density of 100,000 cells/well and incubated overnight for cells to adhere. Chemically synthesized phosphorylated EGFR inhibitors and sorafenib were subsequently added to designated wells to obtain final drug concentration of IC_{50} value obtained from MTT cell viability assay. The treated cells were incubated for 48hrs in 5% CO_2 and 37°C. The supernatant was collected and later pooled together with adhered cells that were detached with 0.5mL of cell dissociation buffer. The cells were washed once in sterile cold PBS and fixed by adding slowly and dropwise 70% ethanol that was kept in -20°C overnight. Fixed cells were kept in the -20°C overnight. Cells were centrifuged and resuspended in PI/RNase Staining buffer (BD, USA) and incubated at room temperature in the dark for 15 minutes (Kalejta *et al*., 1997). Flow cytometry was performed within one hour using the BD Accuri C6 Plus.

2.3.3.4 Expression Of Phosphorylated EGFR

Cultured HCT116 and A549 cells were seeded at a concentration of 500,000 cells/mL on cover slips and placed in 6 well plates overnight. The seeded cells were fixed

in a 4% paraformaldehyde solution and treated with 0.5% triton x to promote permeabilization of the cells. The cells were treated with a blocking buffer and subsequently incubated with the primary antibody (phosphorylated EGFR monoclonal IgG1 mouse antibody (15A2) from Santa Cruz Biotechnology, TX) at 4ºC overnight. The cells were incubated with a secondary Goat Anti-mouse IRDye secondary antibody (LiCor) for 1hr in the dark after which, a drop of Vectashield Antifade Mounting medium with DAPI was placed on each coverslip, inverted unto a microscope slide and observed in a Cytation 3 Cell Imaging Plate Reader.

2.3.4 Statistical Analysis

GraphPad Prism version 8 (San Diego, USA) was used for plotting the graphs and computing the mean and standard deviation of the trials from the experiment. A student t-test was used in comparing mean values and a $p<0.05$ was considered significant.

2.4 Results and Discussion

Removing CASD1 results in the removal of 9-O and 7,9-O acetyl modified sialic acids, indicating a probable way of regulating these modified sialic acids (Barnard *et al*., 2019). Barnard *et al*., further reported that modifying the expression levels of these modified sialic acids influences influenza C and D infection. The role of deacetylated sialic acids in assisting tumors to evade immuno-mediated cytotoxicity by engaging and modulating SIGLECS and their respective ITIMS of immune cells have been proven (Grabenstein *et al*., 2021). However, our results suggests that even with chemotherapy, the lack of SIAE and CASD1 expression in tumor cells, poses some form of chemo-vulnerability on these tumor cells when pEGFR inhibitors are used. All the novel pEGFR inhibitors and sorafenib exhibited a dose-dependent inhibition of cell growth in both the A549 cells and the HCT116 cells. To better understand how the presence or absence of 9-O-acetylation on terminal sialic acids impacts the survival of cancer cells undergoing chemotherapy, we performed a cell viability assay using the MTT dye. To ensure a more targeted cancer therapy, novel small molecules (MMA 294, MMA 320 and MMA 321) that are structural analogs and inhibitors of pEGFR were used as the drug candidates. The data obtained indicated an increase in sensitivity of HCT116 and A549 cells when the CASD1 or SIAE gene is knocked out of the cell (Table 1). This suggests a chemo-protective effect that CASD1 and SIAE proteins might have when they are both present in cancer cells.

Table 2.1 Cytotoxic effect of chemically synthesized phospho-EGFR analogues and Sorafenib on HCT116 and A549 cells.

	HCT116 WT	**HCT116 ΔCASD1**	**HCT116 ΔSIAE**
Sorafenib	9.66 ± 1.58	7.79 ± 0.38	8.36 ± 1.90
MMA 294	8.82 ± 0.87	4.29 ± 0.16	5.25 ± 1.02
MMA 320	8.65 ± 1.14	3.14 ± 0.08	8.43 ± 0.64
MMA 321	12.72 ± 1.07	6.17 ± 1.74	6.97 ± 0.45
	A549 WT	**A549 ΔCASD1**	**A549 ΔSIAE**
Sorafenib	26.74 ± 4.38	9.96 ± 3.41	2.56 ± 1.99
MMA 294	19.07 ± 5.01	4.77 ± 2.60	18.71 ± 4.50
MMA 320	9.02 ± 2.32	6.49 ± 1.67	2.88 ± 0.26
MMA 321	20.97 ± 5.19	1.24 ± 0.09	1.08 ± 0.49

Tabulated values represent Mean IC_{50} ± standard deviation of three independent trials with concentrations in μM

This research suggests a selective mode of cytotoxicity in which cells that are defective in the expression of SIAE and CASD1 are preferentially easily killed. The IC_{50} recorded for all the drugs tested were slightly lower in the knockout cells than the wild type. This is an indication of the fact that the absence of the SIAE and CASD1 proteins in these knockout cells could have made them selectively more vulnerable to cytotoxicity by pEGFR inhibitors. This effect opens up the possibility of a combinatorial targeted chemotherapeutic approach in which CASD1 or SIAE could be targeted with a drug to make cancer cells more vulnerable to a second drug, an EGFR analogue or inhibitor.

Our data on the viability of the cells after they are treated with pEGFR analogues revealed a higher rate of cytotoxicity in ΔCASD1 and ΔSIAE HCT116 and A549 cells. We speculated that this difference that confers a slight level of resistance in the WT cells might be as a result of an altered level of the molecular targets (pEGFR) of the drugs (Deng *et al*., 2017). The presence of fewer molecular targets on the WT could have accounted for

the poor cytotoxicity of the drugs. To investigate this hypothesis, the levels of pEGFR on the cells had to be quantified. An immunofluorescence assay was designed and performed on cover slips. Observation and fluorescence intensity quantification were performed with the Cytation 3 Cell Imaging Plate Reader. Upon screening the levels of pEGFR expressed in the cells, it was revealed that both ΔCASD1 and ΔSIAE cells of A549 and HCT116 have increased expression of this growth receptor.

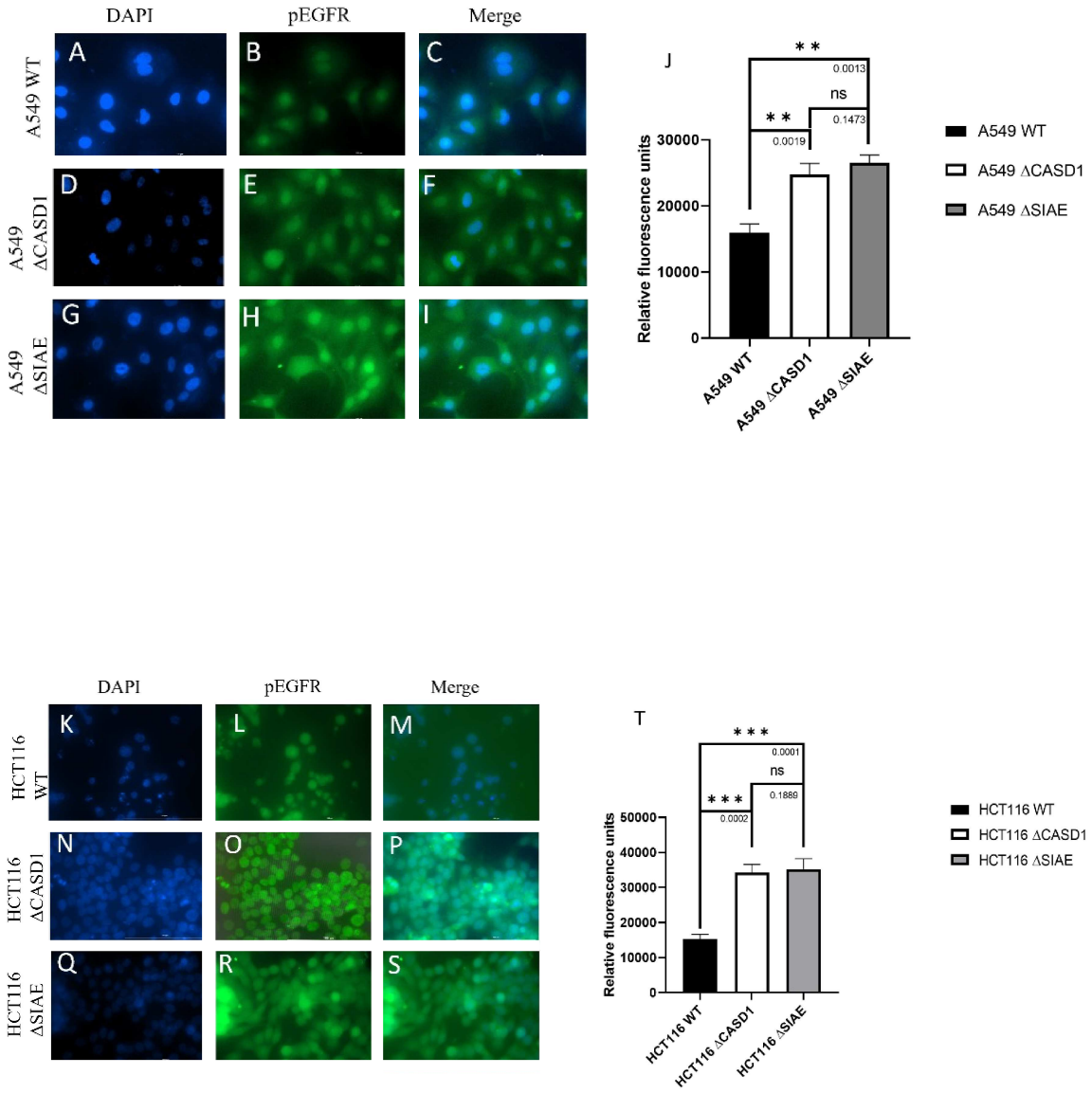


Figure 2.5 Surface distribution of pEGFR on CRISPR Cas-9 gene edited cell lines. Cells were stained for the presence of pEGFR using monoclonal IgG1 mouse antibody primary antibodies overnight at 4◦C and further conjugated with a Goat Anti-mouse IRDye secondary antibody. (A-C) Surface staining of phosphorylated EGFR in A549 WT cells show a reduced level of expression of pEGFR when compared to CRISPR Cas-9 gene edited ΔCASD1 (D-F) and ΔSIAE (G-I). A similar trend observed in HCT116 cells shows an increase expression of pEGFR in ΔCASD1 (N-P) and ΔSIAE (Q-S) than WT (K-M). (J&T) Quantification of the average fluorescence intensity observed shows a significant increase in pEGFR expression when CASD1 and SIAE are knocked out of A549 (J) and HCT 116 cells (T)

Several studies have revealed that the overexpression of EGFR is a major factor in poor tumor prognosis, associated with a more aggressive clinical progression in lung,

breast, ovarian, bladder, oesophageal, cervical and head and neck cancers (Hendler et al., 1988; Richard *et al*., 1987; Neal *et al*., 1985). The increased expression of EGFR in the CASD1 and SIAE knock out cells of A549 and HCT116 as compared to the wild type cells could be indicative of a more aggressive tumor type. As such knocking out CASD1 and SIAE genes could make cancer cells highly proliferative, very aggressive and potentially increase their chances of metastasis due to the increased expression of growth receptors in these cells. However, this newly developed trait could also render these ΔCASD1 and ΔSIAE cells very susceptible to cytotoxicity by pEGFR inhibitors. The increased expression of these growth receptors ensures there are relatively more potential targets for the pEGFR inhibitors and this could account for the much lower IC_{50}s recorded in the knock out cells relative to the wild type cells. Our data supports previous work published by Deng *et al*., 2017 who reported a decrease in IC_{50} concentrations in cells that overexpress EGFR when a targeted EFGR chemotherapeutic agent is used.

The cell cycle is one of the most conserved mechanisms in eukaryotes that is used primarily for replication. Cell cycle distribution of HCT116 and A549 cells were determined after 48hrs of incubation with sorafenib, MMA 294, MMA 320 and MMA 321 and subsequent fixing of cells, treatment with RNase and nuclear staining with propidium iodide. Studies conducted by Zhang *et al*., 2014 showed a G0/G1 cell cycle arrest in A549 that are incubated with sorafenib for 48hrs. In this study, we observed that the distribution of cells in various phases of the cell cycle (G0/G1, S, G2/M) was dependent on the type of drug as well as the type of cell and the genetic composition of the cell type (Figure 2.6 & Figure 2.7).

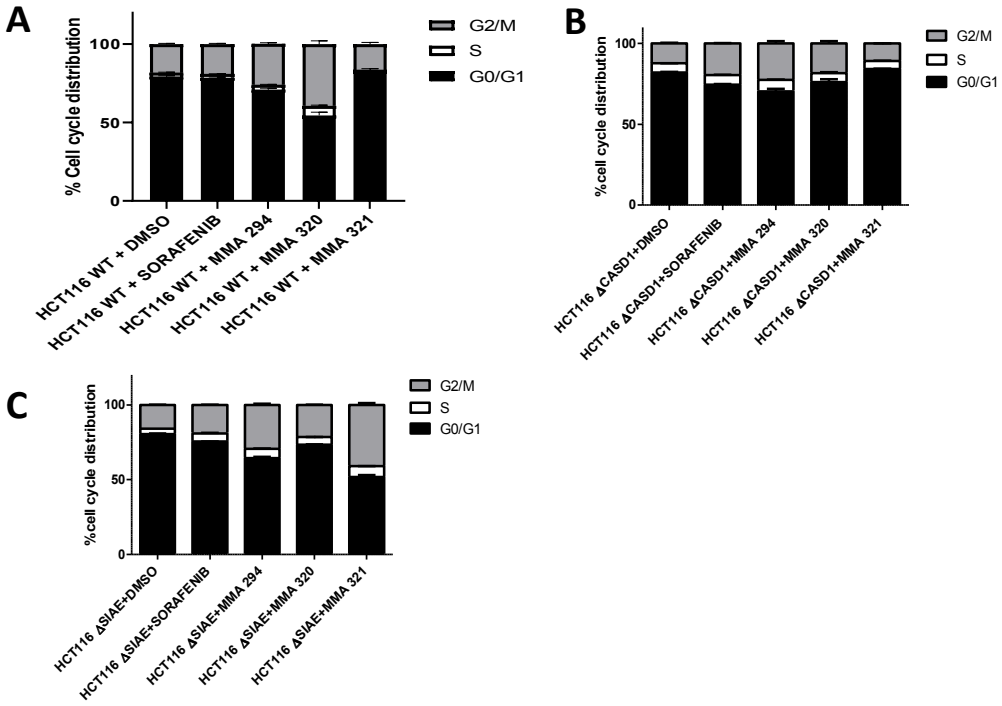


Figure 2.6 Cell cycle distribution of HCT116 A) Wild type B) ΔCASD1 and C) ΔSIAE cells treated with DMSO, Sorafenib, MMA 320, MMA 321 and MMA 294.

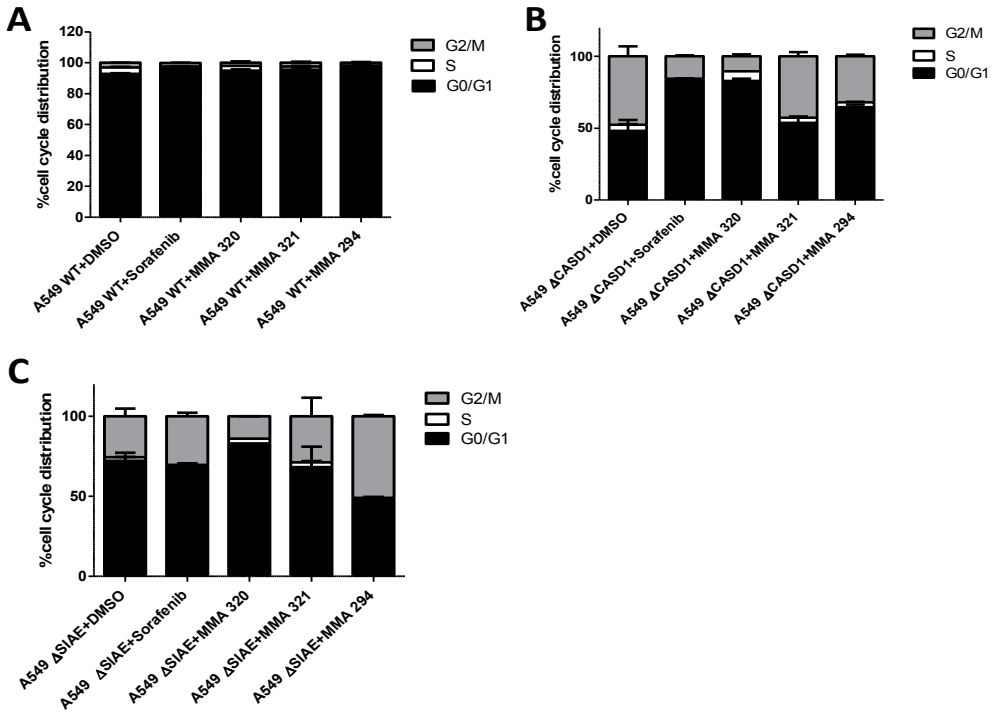


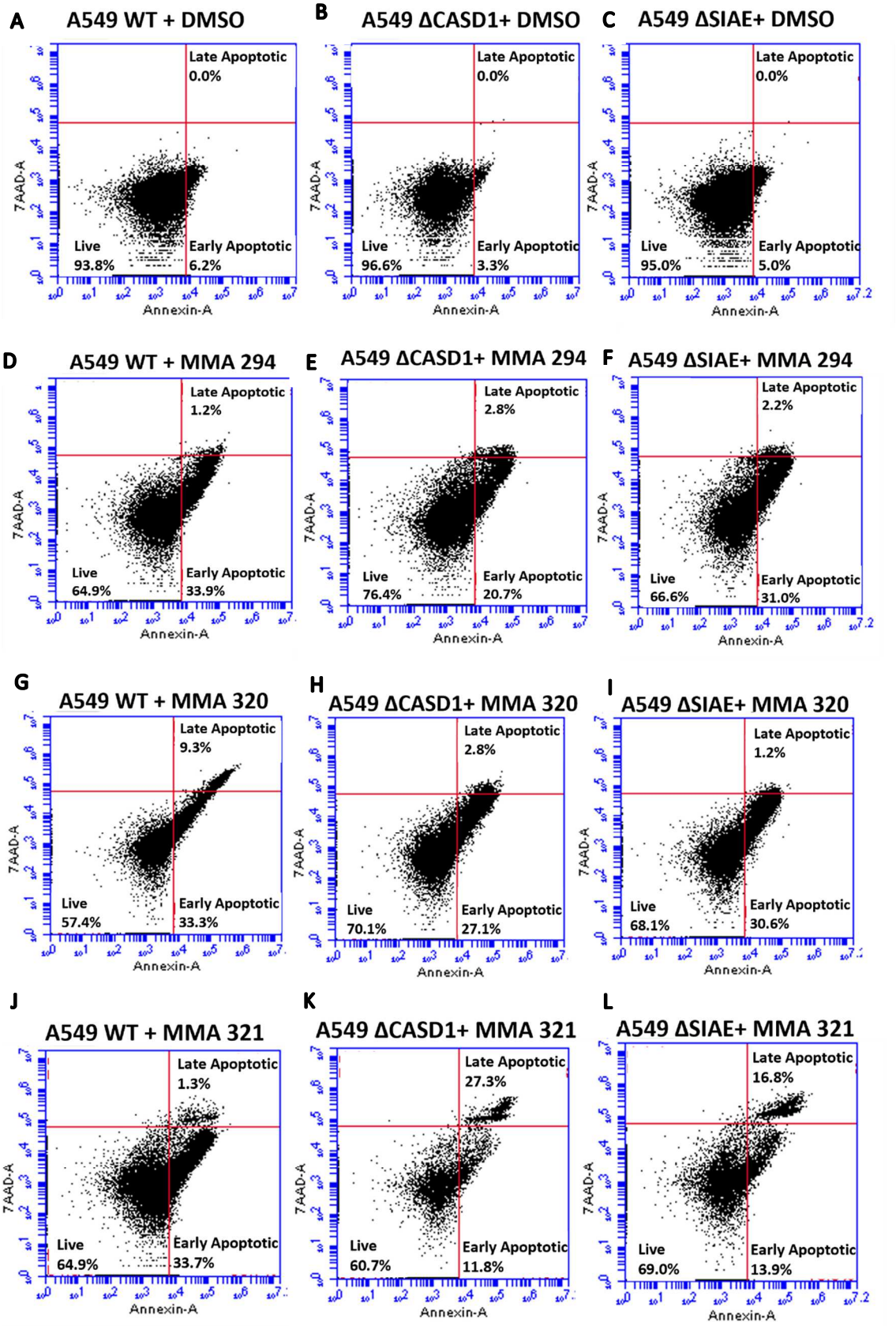
Figure 2.7 Cell cycle distribution of A549 A) Wild type B) ΔCASD1 and C) ΔSIAE cells treated with DMSO, Sorafenib, MMA 320, MMA 321 and MMA 294.

MMA 294, MMA 321 and sorafenib were observed to effect a G0/G1 cell cycle arrest in both A549 WT and HCT116 WT cells. Previous studies on sorafenib revealed that it has multiple targets in cells but arrests HCT116 WT and A549 WT cells at G0/G1 (Plastaras *et al*., 2007; Zhang *et al*., 2014). However, when HCT116 WT cells are irradiated before treatment, Sorafenib arrests cell cycle at G2/M (Plastaras *et al*., 2007). Plastaras et al suggested that exposing the cells to radiation could have caused certain genetic changes within the cells that warranted the switch in the cell cycle arrest profile of the cells. This stands to proof that altering the genetic composition of a cell could cause changes in how it reacts to drugs. Our data suggests that upon treatment of the knockout CASD1 and SIAE cells with these same drugs, there was a switch in cell cycle arrest from the observed G0/G1 in wild type cells to a G2/M cell cycle arrest, confirming the earlier findings by Plastaras et al (2007). This suggests the possibility of a different downstream effector protein in the knockout cells, thus supporting the multiple downstream effectors in cells hypothesis just like sorafenib.

Furthermore, research by Omerod *et al*. (1994) revealed that drugs that arrest at G2/M phase do so at lesser concentrations than those associated with G0/G1. This could explain why the IC_{50}s were lower in the knockout cells relative to the wild type cells of both A549 and HCT116. However, the cell cycle data was not generalizable to adequately account for the observed differences in IC50s since no particular pattern or trend was observed.

Apoptosis is a programmed cell death utilized by cells in controlling tissue or organ development as well as cellular turnover (Koopman *et al*., 1994). The characteristic features of chromatin condensation, cell volume reduction, endonuclease cleavage of

nuclear DNA, loss of nuclear membrane integrity, loss of plasma membrane symmetry and expulsion of phosphatidylserine unto the outer plasma membrane make cells undergoing apoptosis unique (Verhoven *et al*., 1995; Fadok *et al*., 1992). In this study, we successfully utilized two of these key apoptotic features in sorting and characterizing drug treated cells to evaluate whether the mode of cytotoxicity was via apoptosis. Drug treated cells that had been incubated for 48 hrs were treated with APC-Annexin and 7AAD, two well-known reagents that bind to exposed phosphatidylserine (PS) and nuclear DNA to detect cells that were undergoing apoptosis. The mode of cytotoxicity elicited by MMA 294, 320, 321 and Sorafenib was observed to be apoptosis (Figure 1 & Figure 2) which is characterized by the increase in cells found positive for APC-Annexin V only or in combination with 7-AAD.


A
A549 WT + DMSO
Late Apoptotic
0.0%
Live
93.8%
Early Apoptotic
6.2%
B
A549 ΔCASD1+ DMSO
Late Apoptotic
0.0%
Live
96.6%
Early Apoptotic
3.3%
C
A549 ΔSIAE+ DMSO
Late Apoptotic
0.0%
Live
95.0%
Early Apoptotic
5.0%
D
A549 WT + MMA 294
Late Apoptotic
1.2%
Live
64.9%
Early Apoptotic
33.9%
E
A549 ΔCASD1+ MMA 294
Late Apoptotic
2.8%
Live
76.4%
Early Apoptotic
20.7%
F
A549 ΔSIAE+ MMA 294
Late Apoptotic
2.2%
Live
66.6%
Early Apoptotic
31.0%
G
A549 WT + MMA 320
Late Apoptotic
9.3%
Live
57.4%
Early Apoptotic
33.3%
H
A549 ΔCASD1+ MMA 320
Late Apoptotic
2.8%
Live
70.1%
Early Apoptotic
27.1%
I
A549 ΔSIAE+ MMA 320
Late Apoptotic
1.2%
Live
68.1%
Early Apoptotic
30.6%
J
A549 WT + MMA 321
Late Apoptotic
1.3%
Live
64.9%
Early Apoptotic
33.7%
K
A549 ΔCASD1+ MMA 321
Late Apoptotic
27.3%
Live
60.7%
Early Apoptotic
11.8%
L
A549 ΔSIAE+ MMA 321
Late Apoptotic
16.8%
Live
69.0%
Early Apoptotic
13.9%
7AAD-A
Annexin-A

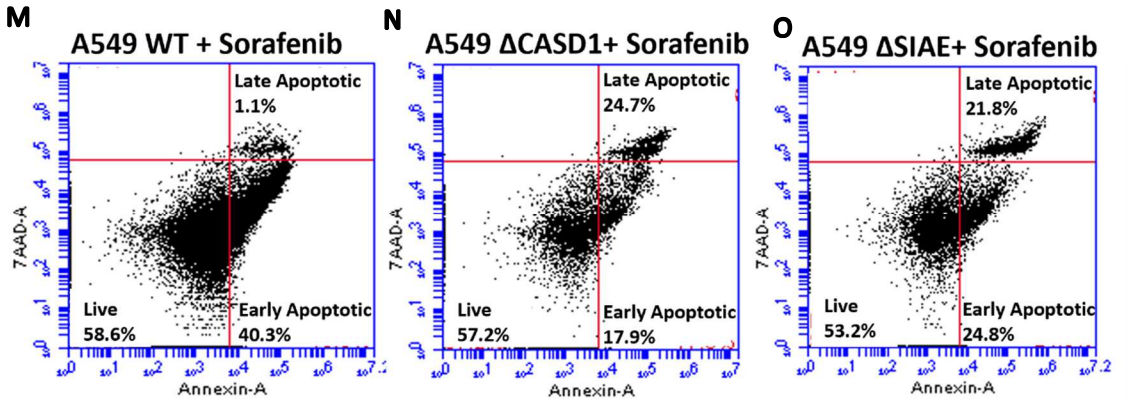
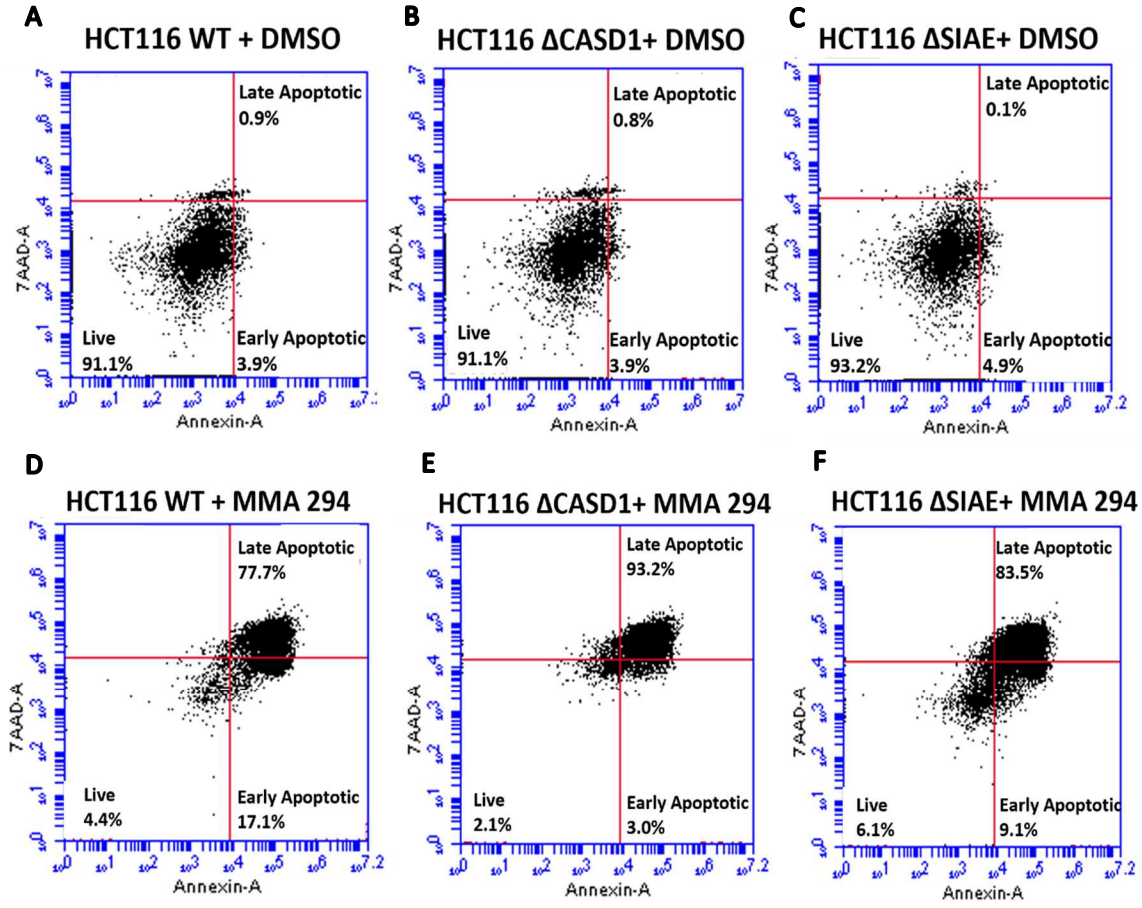


*Figure 2.8 A549 cells treated with novel pEGFR inhibitors MMA 294 (**D-F**), MMA 320 (**G-I**), MMA 321 (**J-L**) for 48 hrs show an increase in cells undergoing early and late apoptosis similar to A549 cells treated with the known anticancer drug sorafenib (**M-O**) relative to the untreated A549 cells (**A-C**).*



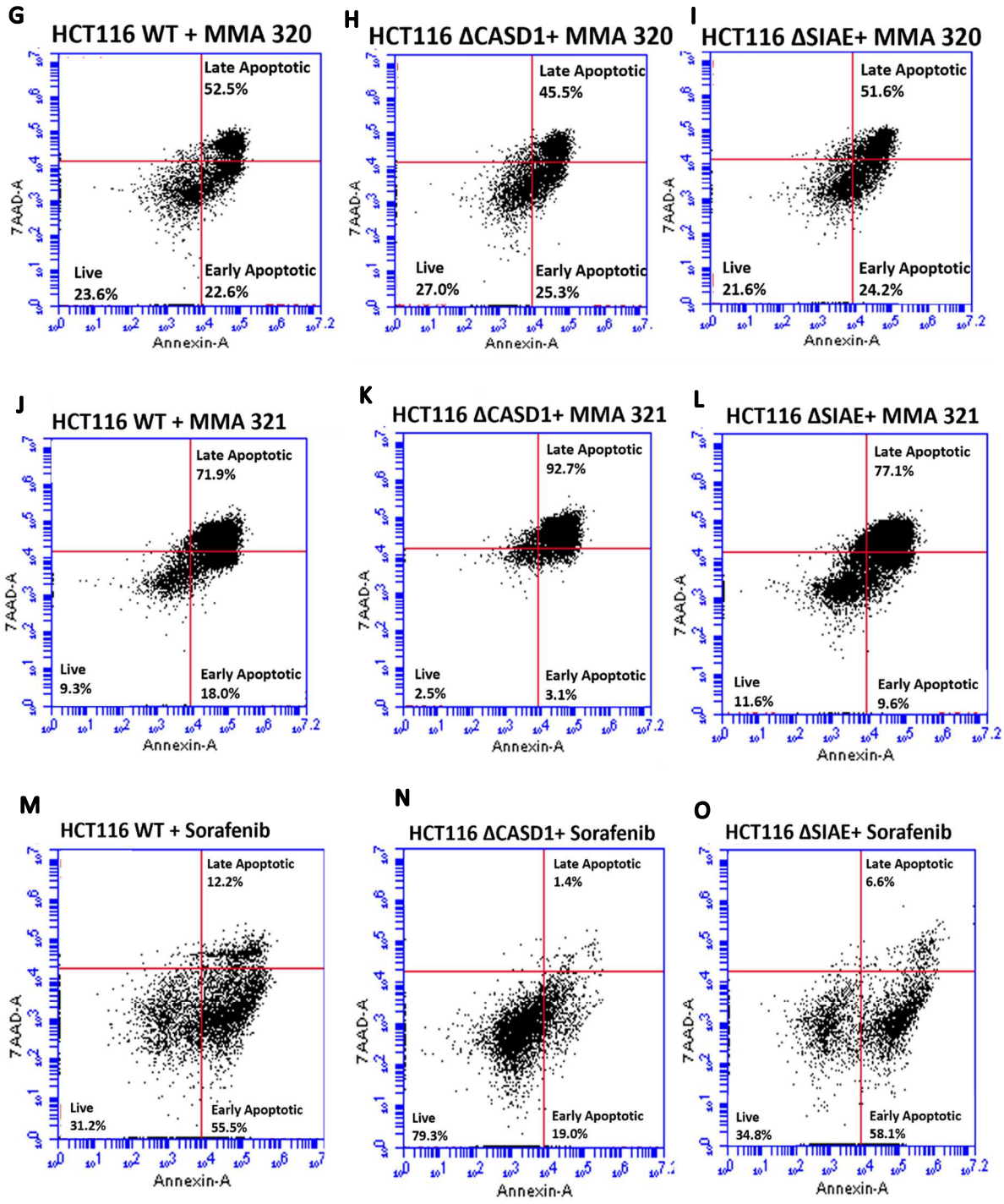


*Figure 2.9 HCT116 cells treated with novel pEGFR inhibitors MMA 294 (**D-F**), MMA 320 (**G-I**), MMA 321 (**J-L**) for 48 hrs show an increase in cells undergoing early and late apoptosis similar to HCT116 cells treated with the known anticancer drug sorafenib (**M-O**) relative to the untreated HCT116 cells (**A-C**).*

Both A549 and HCT116 cells treated with the inhibitors were observed to bind to annexin V and 7AAD both of which can bind to cells undergoing apoptosis. The presence

of phosphatidylserine on the outer plasma membrane and its ability to bind with Annexin V is a hallmark for cells undergoing apoptosis when analyzed with DNA-bound 7AAD (Zhang *et al*., 1997). Though no significant difference was observed between the wild type and knockout cells, the mode of cytotoxicity observed was apoptosis in all the cell lines tested. This makes MMA 294, MMA 320 and MMA 321 good potential chemotherapeutics that could be further explored as drug candidates for a targeted anticancer chemotherapeutic against A549 and HCT116 cells.

2.5 Conclusion

Targeted therapeutics against cancer are increasing in demand and popularity due to their ability to specifically target cancer cells, sparing normal healthy cells. This offers such therapies some advantages over traditional chemotherapeutics. In this study, we have shown that, there is a possibility for improving the effectiveness of targeted therapeutics by selectively targeting a second unrelated protein. More specifically, by targeting glycan modifying proteins like CASD1 and SIAE via CRISPR Cas9 genome editing, lung and colon cancer cells tend to upregulate their EGFR expression levels to compensate for the genetic aberration. This makes these cancers more susceptible to EGFR inhibitors and selectively renders an improved alternative approach through which they can be targeted and killed. This is a multidrug targeted therapy that can be harnessed in cancer treatment.

2.6 References

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