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Advancement of sialyltransferase inhibitors: therapeutic challenges and opportunities

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Abstract

Hypersialylation of tumor cell surface proteins along with a marked upregulation of sialyltransferase (ST) activity is a well-established hallmark of cancer. Due to the critical role of STs in tumor growth and progression, ST inhibition has emerged as a potential new antimetastatic strategy for a range of cancers including pancreatic and ovarian. Human STs are divided into subtypes based on their linkage and acceptor molecule, with each subtype controlling the synthesis of specific sialylated structures with unique biological roles. This has important implications for inhibitor development, as STs also play significant roles in immune responses, inflammation, viral infection, and neurological disorders. Thus, the current goal in order to advance to the clinic is the development of subtype selective, cell-permeable and synthetically accessible, small-molecule ST inhibitors. Herein is a comprehensive review of the latest developments in ST inhibitors from design, Nature, and high-throughput screening, addressing both the challenges and opportunities in targeting cell surface sialylation. The review features an overview of the biological evaluation methods, computational and imaging tools, inhibitor molecular diversity, and selectivity toward ST subtypes, along with the emerging role of ST inhibitors as diagnostic tools for disease imaging.

Keywords

sialyltransferase, inhibitors, therapeutic, advancement, challenges, opportunities

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Advancement of Sialyltransferase Inhibitors: Therapeutic Challenges and Opportunities

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Abstract: Hypersialylation of tumour cell surface proteins along with a marked up-regulation of sialyltransferase (ST) activity is a well-established hallmark of cancer. Due to the critical role of STs in tumour growth and progression, ST inhibition has emerged as a potential new antimetastatic strategy for a range of cancers including pancreatic and ovarian. Human STs are divided into subtypes based on their linkage and acceptor molecule, with each subtype controlling the synthesis of specific sialylated structures with unique biological roles. This has important implications for inhibitor development, as STs also play significant roles in immune responses, inflammation, viral infection and neurological disorders. Thus, the current goal in order to advance to the clinic is the development of subtype selective, cell-permeable and synthetically accessible, small-molecule ST inhibitors. Herein, is a comprehensive review of the latest developments in ST inhibitors from design, Nature and high-throughput screening, addressing both the challenges and opportunities in targeting cell surface sialylation. The review features an overview of the biological evaluation methods, computational and imaging tools, inhibitor molecular diversity and selectivity towards ST subtypes, along with the emerging role of ST inhibitors as diagnostic tools for disease imaging.

Key words: sialyltransferase inhibitors, structure-based design, anticancer agents, antimetastasis, diagnostic tools.

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1. INTRODUCTION

Sialic acid is the body's most important sugar next to glucose.¹ Sialylation, the addition of sialic acid to cell surface molecules via sialyltransferase enzymes, is integral to cell function, governing numerous important biological processes including cell-cell recognition, adhesion, protein targeting and fertilization.² However, aberrant sialylation is a hallmark of cancer, with hypersialylation of up to 30% seen in many cancers, along with marked up-regulation of sialyltransferase activity. This is directly correlated with increased metastatic potential and poor patient prognosis.^{3,4} Sialylation mediates tumour metastasis via several routes including stimulating tumour invasion and migration through integrin-mediated processes,^{5,6} promoting tumour survival by blocking galectin binding,⁷ and inhibiting Fas-mediated apoptosis.^{4,8} Due to the critical role of sialyltransferases (STs) in tumour metastasis, ST inhibition has emerged as a potential new anti-metastatic treatment for several cancers.⁹

There are 20 human STs divided into four groups based on their linkage type and acceptor molecule: ST3Gal I-VI; ST6Gal I-II; ST6GalNAc I-VI; and ST8Sia I-VI.¹⁰ Each ST controls the synthesis of specific sialylated structures with unique biological roles, such as Lewis antigen sialylation by ST3Gal III, IV and VI and β 1 integrin sialylation by ST6Gal I.¹¹ Thus, it is critical for the advancement of this field that selective inhibitors are used to unravel the roles of individual STs in tumour growth, metastasis and disease progression and for the development of a new treatment strategy for a range of treatment resistant cancers including pancreatic and ovarian cancer. Furthermore, resistance to frontline therapies such as paclitaxel and the platinum-based drugs cisplatin and carboplatin, is a critical issue in cancer treatment resulting in significant disease relapse, and reducing patient survival rates. Upregulation of STs has been shown to produce cellular resistance in both paclitaxel and cisplatin treatment in human ovarian and colorectal cancer cell lines along with reducing treatment efficacy.¹² This demonstrates that small molecule inhibitors of STs could be used either alone as a new therapeutic treatment for selected cancers, or in combination with existing drugs to enhance their sensitivity in resistant tumours.

Herein, we review the latest achievements in the development of ST inhibitors by design, from Nature and from high-throughput screening (HTS), along with examining the emerging role of ST inhibitors as biological tools for cancer imaging.

A. Sialic Acid

Sialic acids belong to a family of sugars containing a nine-carbon backbone bearing a carboxy group at the 1-position, a hydroxy group at the 2-position and an amino group at the 5-position, with over 50 derivatives known.^{1,13} *N*-Acetylneuraminic acid, Neu5Ac (**1**) is the most ubiquitous and well known member of this family alongside other important members including the 5-glycolylamido derivative Neu5Gc (**2**) and the non-aminated derivative 2-keto-3-deoxynononic acid, KDN (**3**) (Fig. 1).^{2,14} Higher animals and some microorganisms biosynthesize sialic acids from ManNAc for use in many reactions. Ubiquitous components of mammalian glycoproteins and glycolipids, they are a family of closely related neuraminic acids found at the non-reducing terminal positions of glycoconjugates, usually attached to the exposed terminus of *N*- and *O*-linked glycans of glycoproteins. Sialic acid thus represents one of the most important constituents of glycoconjugates in biological systems.^{1,2,14}

Figure 1. Structures of selected sialic acids.

Due to their terminal position and their negative charge at physiological pH, sialylated oligosaccharide sequences are key information containing molecules and critical determinants in cell-cell recognition processes, cell-matrix interactions and maintenance of serum glycoproteins in the circulation.^{15,16} The cell surface sialic acid residues have been known to act as receptors for the influenza virus.¹⁷ They often function in the “anti-recognition” or masking of the carbohydrate groups they terminate.¹⁶ The highest concentration of sialic acid (as Neu5Ac) in the human body occurs in the brain where it is an integral part of ganglioside structure. Consistent with this finding, human milk contains a high concentration of sialic acid, which is associated with the increase of gangliosides in the brain and is potentially linked to improved learning ability.¹⁸ Wang and Brand-Miller have reviewed the role of sialic acid in human nutrition and the link between sialic acids, breastfeeding and learning behaviour.¹⁹ Conversely, a reduction in sialic acid is correlated with erythrocyte destruction.²⁰

B. Sialyltransferases

Sialyltransferases are a subset of glycosyltransferases (GTs) involved in the biosynthesis of sialylated glycolipids and/or glycoproteins. Sialyltransferases catalyse the addition of sialic acid residues from the β -glycoside donor cytidine monophosphate *N*-acetylneuraminic acid,

CMP-Neu5Ac (**4**) (Fig. 2) to the terminal position of growing oligosaccharide chains of glycoconjugate acceptors, to produce α -sialosides. In vertebrates, CMP-Neu5Ac is produced in the cell nuclei and transported to the Golgi apparatus where sialylation takes place.²¹⁻²³ Depending on the glycosidic linkage formed and their glycoconjugate acceptor, vertebrate STs are arranged into four families (ST3Gal, ST6Gal, ST6GalNAc and ST8Sia; Table I). STs families are further subdivided into 20 sub-types in mammals that are highly conserved from mouse to man²⁴ and more than 25 sub-types in lower vertebrates, each of them being characterized by conserved amino acid positions. All utilise CMP-Neu5Ac as the activated sugar donor to catalyse the transfer of sialic acid residues to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids.^{25,26}

Figure 2. Mechanism of the sialylation reaction and expected products by sialyltransferase subtypes.

STs differ in their substrate specificity, tissue distribution and various biochemical parameters. Enzymatic analysis conducted *in vitro* with recombinant enzyme revealed that one linkage can be synthesised by multiple enzymes.^{27,28} Moreover, even though eukaryotic STs share the same sugar donors, *i.e.* CMP-Neu5Ac (**4**), and recognize identical acceptor substrates, they do not exhibit similar protein structure except for the four short and conserved consensus sequences present in the catalytic domain.^{29,30} STs have been shown to be primarily topologically restricted to trans cisternae and the trans Golgi network of the

Table I. Nomenclature of the 20 human sialyltransferases (STs) described to-date.³⁷⁻³⁹

Abbreviation	Substrates	EC number	HGNC ^a	Older names	Gene ID
ST3Gal I	Gal- β -1,3-GalNAc	2.4.99.4	10862	SIAT4A ST3O	8q24.2 (6482)
ST3Gal II	Gal- β -1,3-GalNAc	2.4.99.4	10863	SIAT4B	16q21-22.3 (6483)
ST3Gal III	Gal- β -1,3(4)-GlcNAc	2.4.99.6	10866	SIAT6 ST3N	1p34.1 (6487)
ST3Gal IV	Gal- β -1,4-GlcNAc	2.4.99.9	10864	SIAT4, SIAT4C CGS23, FLJ11867, NANTA3, STZ	11q23-q24 (6484)
ST3Gal V	Gal- β -1,4-Glc	2.4.99.9	10872	SIAT9 GM3 synthase	2p11.2 (8869)
ST3Gal VI	Gal- β -1,4-GlcNAc	2.4.99.9	18080	SIAT10	3q12.2 (10402)
ST6Gal I	Gal- β -1,4-GlcNAc	2.4.99.1	10860	SIAT1 MGC4885	3q27.3 (6480)
ST6Gal II	Gal- β -1,4-GlcNAc	2.4.99.2	10861	SIAT2 KIAA1877	2q12.3 (84620)
ST6GalNAc I	GalNAc- α -1, O-Ser/Thr	2.4.99.7	23614	SIAT7A	17q25.3 (55808)
ST6GalNAc II	Gal- β -1,3-GalNAc- α -1, O-Ser/Thr	2.4.99.7	10867	SIAT7, SIAT7B STHM, ISTHM	17q25.1 (10610)
ST6GalNAc III	Neu5Ac- α -2,3-Gal- β -1,3-GalNAc	2.4.99.7	19343	SIAT7C	1p31.1 (256435)
ST6GalNAc IV	Neu5Ac- α -2,3-Gal- β -1,3-GalNAc	2.4.99.7	17846	SIAT7D, SIAT3C	9q34 (27090)
ST6GalNAc V	Neu5Ac- α -2,6-GalNAc- β -1,3-GalNAc	2.4.99.7	19342	SIAT7E MGC3184	1p31.1 (81849)
ST6GalNAc VI	All α -series gangliosides	2.4.99.7	23364	SIAT7F	9q34.13 (30815)
ST8Sia I	Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -1,1Cer (GM3)	2.4.99.8	10869	SIAT8, SIAT8A GD3 synthase	12p12.1-p11.2 (6489)
ST8Sia II	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc	2.4.99.8	10870	SIAT8B HsT19690, MGC116854, STX	15q269 (8128)
ST8Sia III	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc	2.4.99.8	14269	SIAT8C	18q21.31 (51046)
ST8Sia IV	(Neu5Ac- α -2,8) _n Neu5Ac- α -2,3-Gal- β -1-R	2.4.99.8	10871	SIAT8D PST, PST1, MGC34450, MGC61459	5q21 (7903)
ST8Sia V	GM1b, GT1b, GD1a, GD3	2.4.99.8	17827	SIAT8E	18q12.3 (29906)
ST8Sia VI	Neu5Ac- α -2,3(6)-Gal	2.4.99.8	23317	SIAT8F	10p13 (338596)

^a HGNC: Hugo Gene Community Nomenclature

Golgi apparatus, although catalytically active soluble forms can be generated *in vivo* by proteolytic cleavage at the stem region, *e.g.* serum soluble ST6Gal I.³¹ The existence of cell surface ST activity has also been reported for human monocyte-derived dendritic cells.³² Due to the paucity of efficient selective, cell permeable inhibitors, much of the available information regarding the importance of sialyltransferases has come from gene ablation in mice.^{33,34} Recent research has led to further understanding of the biological involvement of these enzymes in a host of disease states, further extending the array of potential therapeutic applications of this target.^{35,36}

C. Sialylation in Cancer

Elevated plasma levels of STs have been observed in cancer patients since the 70's, and hypersialylation of cell surface proteins is now a well-established hallmark of cancer that forms the basis of several diagnostic cancer markers.⁴⁰⁻⁴² One such example is the carbohydrate antigen CA19-9 for pancreatic cancer, which results from overexpression of ST3Gal III, IV and VI leading to the production of large amounts of sialyl Lewis^a (sLe^a). Sialylated glycans have long been predicted as cell-type specific markers and critical determinants in various cellular recognition processes (cell–cell recognition, adhesion, protein targeting, blood coagulation, fertilization, and other biological events).⁴³ They also play significant roles in immune responses,⁴⁴ inflammatory diseases, viral infection^{45,46} and tumour proliferation,⁴⁷ migration, and metastasis.⁴⁸⁻⁵⁰ STs have been described as “engines of self-fuelling loops in cancer progression”.^{4,8,51} Hypersialylated integrins are believed to facilitate cell migration in many different cancer types,⁵² such as colon,^{53,54} lung^{55,56} and ovarian cancer.¹¹ Aberrant sialylation at the non-reducing end of glycoproteins or glycolipids has been shown to promote events involving abnormal stimuli in the interaction of neoplastic cells with extracellular matrices.⁹ The biosynthesis of sialylated glycoprotein, mediated by ST6GalNAc IV, was also reported on various kidney cancer cell lines.⁵⁷ Before considering membrane sialylated glycoproteins as a target for treating cancer, these molecular patterns are useful in the prognosis of cancer^{58,59} and other diseases.⁶⁰ Tumour cell migration and invasion are regulated by sialylated glycoforms of integrins. Specifically, β 1 in colon adenocarcinomas^{53,54} and lung,^{55,56} where ST expression, in particular ST6Gal I,⁶¹ ST3Gal I,^{62,63} ST3Gal III,^{48,61} ST3Gal IV,^{48,64} ST6GalNAc I⁶⁵ and ST6GalNAc V⁶⁶ are strongly correlated with tumour invasiveness. This is also the case in numerous other cancers including, bladder,⁶³ breast,⁶⁷ pancreatic,⁶⁸ ovarian,¹¹ gastro-intestinal⁶⁹ cancers and leukemia.⁷⁰ (Fig. 3).

Lung Cancer: Sialyl Lewis x (SLe^x) is a well-known tumour-associated antigen that is found in elevated levels in lung cancer. It facilitates cell adhesion to the endothelium, initiating the process of extravasation and metastasis. ST3Gal III, IV and VI enzymes play a key role in the synthesis of SLe^x. In lung cancer, expression levels of both SLe^x and ST3Gal III are inversely correlated with patient survival.⁷¹ Hypersialylation and increased expression of SLe^x is also observed in the mucins of cystic fibrosis patients,⁷² while over-sialylation of several other proteins including integrins has also been shown to enhance cell invasion in ovarian, colon and lung cancer. ST3Gal I is not detected in normal human bronchial epithelial cells, but is over-expressed in highly metastatic lung cancer cells such as A549 and CL1-5.⁵⁶ Thus, inhibition of STs, in particular the ST3Gal subfamily, has emerged as a new target for the treatment of early lung cancer, and to aid in understanding the progression to more aggressive phenotypes.

Ovarian Cancer: Increased serum glycoprotein sialylation in a murine ovarian carcinoma model has been found to correlate with inflammation and tumour progression, with increased expression of ST3Gal I and ST6Gal I.⁷³ The latter also regulates ovarian tumour cell survival mechanisms⁷⁴ and confers cisplatin resistance.^{75,76} CA125, the clinically used ovarian cancer marker is a heavily glycosylated mucin (MUC16).⁶² Levels of sialyl-Tn antigen, expressed on MUC16 in patients, is found to differ between endometriosis and ovarian cancer raising the potential for its use in diagnosis, and evaluating the clinical stage, cytological grade, and histological type of ovarian cancer.⁷⁷

Breast Cancer: STs have been found to play a key role in breast cancer progression, aggressiveness, metastasis and drug resistance. Recent studies in triple-negative breast cancer patient-derived xenograft models treated with standard chemotherapy identified high levels of the sialyl-glycolipid stage-specific embryonic antigen 4 (SSEA4). This treatment-resistant breast cancer subpopulation also exhibited high expression of ST3Gal II, which is required for the biosynthesis of SSEA4. This could be used as a potential predictive biomarker of poor clinical outcomes in both breast and ovarian cancer patients treated with standard-care genotoxic chemotherapy.⁷⁸ Gene expression analysis coupled with functional analysis of clinical samples has also identified ST6GalNAc V as a key mediator of breast cancer metastasis to the brain. Atypical expression of this brain ST in breast cancer cells is thought to facilitate their passage through the blood-brain barrier.⁷⁹

The transmembrane tumour-associated glycoprotein Mucin 1 (MUC1) is aberrantly glycosylated and overexpressed in a variety of epithelial cancers, where it is involved in intracellular signal transduction pathways.⁸⁰ A strong correlation between the expression of

MUC1 and T antigen has been found in breast cancer tumours and breast cancer cell lines, along with the down-regulation of expression of core 2 β -1,6-N-acetylglucosaminyltransferase (C2GnT1) and ST3Gal I.⁸¹ Cyclooxygenase-2 (COX-2) is involved in the upregulation of ST3Gal I in MDA-MB-231 breast cancer cell lines leading to increased sialylation of the ST3Gal I substrate, core 1 Gal- β -1,3-GalNAc, which can be reversed using the COX-2 inhibitor celecoxib. Expression analysis of 74 primary breast cancers showed a significant correlation between ST3Gal I and COX-2, indicating that malignant characteristics of COX-2 expression may be related to tumour cell surface sialylation.⁸² On a different note, cationic amphibian antibacterial peptides such as aurein 1.2 from the glands of the *Litoria* sp. frog and buforin IIb derived from the Asian toad *Bufo garagrios*, exploit the upregulation of sialylation in breast cancer cells (MX-1 and MCF-7) to enhance their binding to sialylated *N*-, *O*-glycoproteins and gangliosides on the tumour cell surface. This may contribute to their selective cancer toxicity. Buforin IIb also suppressed tumour growth in a MX-1 xenograft mouse model via induction of apoptosis and inhibition of vascularization.⁸³

Multiple Myeloma: High expression of ST3Gal VI has been identified in patients with multiple myeloma (MM) and correlated to poor patient prognosis. Knockdown of ST3Gal VI in MM cell lines MM1S and RPMI-8226 significantly reduced α -2,3-linked sialic acids on the cell surface and reduced adhesion to bone marrow stromal cells and fibronectin. It further inhibited transendothelial migration *in vitro* and reduced homing and engraftment, decreased tumour burden and increased survival *in vivo* in a xenograft (SCID-Bg) mouse model.⁸⁴

Leukemia and Glioma: Elevated mRNA levels of ST expression have been found to positively correlate with a high risk of numerous cancers including paediatric leukemia, where it has been suggested as a potential biomarker.⁸⁵ In particular, cell surface sialylation is emerging as an important feature of cancer cell multidrug resistance (MDR). Recently, the α -2,8-sialyltransferases ST8Sia IV and ST8Sia VI were found to positively correlate with the multidrug resistant phenotype in chronic myeloid leukemia (CML) cells, with ST8Sia IV appearing to regulate the activity of PI3K/Akt signalling and P-gp expression.^{86,87} Overexpression of ST6GalNAcV, a ganglioside-specific α 2,6-sialyltransferase, inhibits glioma growth *in vivo*.⁸⁸

Metastasis and MDR: Also of important consideration is metastasis, a major challenge in the treatment of cancer, largely responsible for the high rate of cancer mortality. It is emerging that molecules conferring drug resistance may also promote metastatic invasion. This gives rise to the situation where metastatic disease is often more resistant to treatment.¹²

Cancers where ST over-expression has been identified as playing a critical role in cancer progression include colon, pancreatic and prostate cancer.⁸⁹

Figure 3. Gene alteration frequency of sialyltransferase subtypes in various cancers.⁹⁰

D. Central Nervous System

In contrast to the aberrant hypersialylation that is a hallmark of many cancers, a range of neurological and memory disorders including Alzheimer's,⁹¹⁻⁹⁴ schizophrenia,⁹⁵⁻⁹⁸ autism,⁹⁹ bipolar disorder,¹⁰⁰ depression,¹⁰¹ chronic stress¹⁰² and alcoholism,¹⁰³ exhibit a hyposialylation phenotype, along with a concomitant down regulation of various sialyltransferases. The ST8Sia II gene has been identified as a generalized susceptibility gene for psychotic and mood disorders,^{104,105} as demonstrated by McAuley et al. using genome wide association study (GWAS).¹⁰⁰ It has also found to be associated with schizophrenia in Spanish,¹⁰⁶ Japanese,¹⁰⁷ and Chinese Han populations,^{95,96} along with an association with autism,⁹⁹ depression,¹⁰¹ and an increased risk of bipolar spectrum disorder in specific patient and familial cases.^{108,109} Inhibition of brain ST8Sia III has been found to lead to impairment of procedural memory in mice.¹¹⁰ Polysialic acids were also reported to play a role in the development of neurons,¹¹¹ dendritic cell trafficking¹¹² and in the reset of the circadian clock.¹¹³

Thus, the role and function of reduced sialylation in memory and neurological disorders will need to be further explored alongside the development of small molecule ST inhibitors directed at hypersialylation in diseases such as cancer. This also highlights the importance of

developing inhibitors that are selective for particular ST-subtypes, along with exploration of targeted delivery of ST inhibitors directly to the tumour site.

E. Immune System, Inflammation and Others

Not only are STs an attractive target for cancer, they also present interesting applications in other biological fields. The ST6Gal I sialyltransferase controls production of the Sia- α -2,6-Gal- β -1,4-GlcNAc (Sia6LacNAc) trisaccharide, which is the ligand for the lectin CD22. Binding of CD22 to Sia6LacNAc is implicated in regulating lymphocyte adhesion and activation. Knock-out ST6Gal I mice were found to be viable and thus constitute a good model for immunodepression, unlike CD22-deficient mice. Human sialoconjugates incorporate the Neu5Ac form of Sia in contrast to Neu5Gc in mice, which is the main difference.¹¹⁴ Notably, Sia6LacNAc-deficient mice displayed reduced serum IgM levels, impaired B cell proliferation in response to IgM and CD40 crosslinking, and attenuated antibody production to T-independent and T-dependent antigens. Deficiency of ST6Gal was further found to alter phosphotyrosine accumulation during signal transduction from the B lymphocyte antigen receptor. These studies reveal that the ST6Gal I sialyltransferase and corresponding production of the Sia6LacNAc oligosaccharide are essential in promoting B lymphocyte activation and immune function.³⁴ Other disease states linked to a loss of ST gene function include allergies, where deficiencies in ST6Gal I and ST3Gal III production in mice is associated with enhanced allergic airway inflammation.^{115,116}

Leukocytes are activated by binding to α -2,3-sialylated glycans and as a result, promotes immunodepression due to a deficiency in the ST3Gal IV and VI enzymes.¹¹⁷ Leukocyte accumulation was also shown to play a key role in atherosclerosis. In this field, ST3Gal-IV has been identified as a promising target to reduce inflammatory leukocyte recruitment. Studies by Doering *et al.* have shown ST3Gal-IV deficiency leads to reduced chemokine Ccl5 binding and Ccl5-induced flow arrest of myeloid cells, with marked reduction in the inflammatory cell content of lesions in a mouse model of atherosclerosis.¹¹⁸ Rifat *et al.* also investigated the influence of α -2,6-sialylated glycans on modulating the immunological characteristics of neutrophil granulocytes.¹¹⁹

It has recently been shown that the immune killer cells' activating receptors (NKG2D and CD94) bind to α -2,3-sialylated, but not to α -2,6-sialylated *N*-glycans.¹²⁰ Reduction in cytotoxic T cells (CD8+) had been previously observed in mice after ablation of the ST3Gal I gene. Sialylation clearly plays a key role in the immune system¹²¹ and thus ST inhibition is also a promising therapeutic strategy against inflammation.¹²²

As avian influenza viruses preferentially use α -2,3-linked sialic acid as a receptor for binding and entry into target cells, the selective inhibition of ST3Gal IV represents a potential target for antiviral drug design.¹²³ Another study identified the implication of ST6Gal I in the infection of epithelial cells by viruses pdmH1N1 and H3N2.⁴⁵ The myxoma virus was previously shown to express α -2,3-STs and the resulting sialylated glycans promote infection.¹²⁴ Cells infected with HIV were found to contain higher levels of STs than healthy cells.¹²⁵ Sialylated glycoconjugates also constitute an important platform for host cell invasion for parasites, such as infection with *Toxoplasma* parasites.¹²⁶ The Gram-negative bacteria *Neisseria gonorrhoeae* expresses an α -2,3-sialyltransferase (Lst) that scavenges sialic acid from the host and uses it to sialylate the terminal lacto-*N*-neotetraose moiety of its lipooligosaccharide (LOS). The resulting LOS sialylation is linked to serum resistance, decreased antibody binding and decreased effectiveness of antimicrobial peptides, and thus represents a potential new target for gonorrhea.¹²⁷

ST6Gal I^{128,129} and ST8Sia I¹³⁰ are both downregulated in the liver by chronic alcohol consumption, along with reduced sialylation of serum transferrin and apolipoprotein E (apoE).¹³¹ This affects its ability to bind to high density lipoprotein (HDL) leading to impaired reversed cholesterol transport in alcoholics.¹³² *In utero* alcohol exposure is also found to alter the expression and sialylation of NCAM isoforms during brain development in rats.¹³³

Considerable alteration of ST8Sia II was observed in patients who received gastric bypass surgery.¹³⁴ GWAS was conducted on 693 participants and showed reduced expression of the gene in the liver and epididymal fat and revealed a significant association of the ST8Sia II gene expression with weight loss.

The altered ST expression in a wide range of diseases highlights the critical and specific role that different ST subtypes play in human biology. Furthermore, it illustrates the growing need to develop selective inhibitors in order to further explore the intricate role of sialylation in human disease.

F. Reviews

The design and development of potent small molecule ST inhibition was pioneered by R.R. Schmidt and co-workers,¹³⁵⁻¹³⁸ almost two decades ago. The synthesis and biological evaluation of ST inhibitors appearing at that time have been reviewed previously and focussed on α -2,3-ST and α -2,6-ST subtypes.¹³⁹⁻¹⁴² In the intervening years since the first development of ST inhibitors, a rising number of biological and medical-related papers

have appeared. This has expanded our understanding of the role of glycosylation in various disorders, as well as the role of altered sialylation in disease, in particular cancer and metastasis.¹⁴³ For further reading in this area, there are several comprehensive reviews on the biological function of sialic acids by Schauer & Kelm,^{16,144,145} Varki^{1,2,36,146} and Crocker,^{147,148} and on the biological role of sialylation including reviews by Dall'Olio *et al.*,^{3,51,149} Schultz *et al.*,⁸ Harduin-Lepers *et al.*,²⁶⁻²⁸ and Boons *et al.*¹⁵⁰

Over the last decade, the therapeutic potential of targeting STs has appeared in part in reviews focusing on GTs and altered glycosylation in cancer from Brooks *et al.*,^{151,152} Brown *et al.*,¹⁵³ Compain & Partin,¹⁵⁴ Gloster & Vocadlo,¹⁵⁵ Hinou & Nishimura,¹⁵⁶ Izumi *et al.*,¹⁵⁷ Kajimoto & Node,¹⁵⁸ Roychoudhury & Pohl,¹⁵⁹ Schnaar *et al.*¹⁶⁰ and Zhou *et al.*¹⁶¹ The last comprehensive small molecules review of ST inhibitors appeared over a decade ago, before the first ST crystal structures were reported.^{139,140} In recent years, along with the mounting evidence of the critical role of sialylation in cancer and growing structural information on STs, several new potent ST inhibitors have been described. These have been derived from various scaffolds and thus increasing the molecular diversity of ST inhibitors in the literature. Herein, we review the latest highlights in the design, development, biological evaluation and applications of ST inhibitors in both medicinal chemistry and as important tools in chemical biology. This review draws on the recent structural information available for STs, with a particular emphasis on the challenges in the field, such as selectivity of inhibitors toward the different ST subtypes, cell permeability, synthetic practicality, structure-based design and the emerging picture of structure-activity relationships.

2. BIOLOGICAL ASSAYS

A. Enzyme Assays

A key challenge in the ST inhibitor field is the choice of biological assay employed to evaluate the effectiveness of newly developed inhibitors. There are no commercially available ST inhibitor screening assays at the time of writing, and very few commercial sources of mammalian ST enzymes, those being rST3Gal I (EC 2.4.99.4) from Merck (#566227) and hST6Gal I (EC 2.4.99.1) from Merck (#566223) and Roche (#07-012-250-103). For this reason, most research groups clone, express and/or isolate their own ST enzymes, which are used in either crude, partially purified or purified form.^{27,162-170} As with all enzyme work, there are a wide range of enzymes used (e.g. predominantly human,

rat, and bacterial STs) and a variety of assays employed (colorimetric, fluorescent, radioactive, mass spectrometry and HPLC-based) to evaluate inhibitory activity, and this should be considered when comparing biological results. Herein, a brief description of the bioassays employed in the evaluation of ST inhibitors is included below, and in later sections the assay utilised by each research group is highlighted for each study.

HPLC-based Assays: Earlier studies into sialyltransferases used asialofetuin or lacto-*N*-tetraose as the acceptor moiety and ^{14}C or ^3H -labelled CMP-Neu5Ac¹⁷¹ as the donor and the radioactive products were isolated by chromatography or precipitation.¹⁷¹⁻¹⁷³ In 1998, RR Schmidt and co-workers introduced a HPLC-based assay using the *p*-nitrophenyl glycoside of *N*-acetyllactosamine as a UV labelled acceptor, and this continues to be one of the most widely used ST inhibition assays today.¹³⁵ Recent extensions of this method, include a continuous all-in-one HPLC-based assay that enables the quantification of productive turnover, error hydrolysis and site selectivity of sialyltransfer and donor substrate hydrolysis, with the potential for high-throughput screening.¹⁷⁴ Further HPLC-based assays include those reported by Kijahara *et al.* and involve a combination of HPLC analysis with a fluorescent analytical technique based on reacting sialic acid with 1,2-diamino-4,5-methylenedioxybenzene,¹⁷⁵ or using 2-[(2-pyridyl)aminoethyl- β -D-lactosamidine as a ST acceptor for potential use in fluorescent assays.¹⁷⁶

Fluorescence-based Assays: Rillahan *et al.* designed a fluorescence polarisation (FP) assay enabling high-throughput screening of sialyl- and fucosyltransferases.¹⁷⁷ The method employs a CMP-Neu5Ac derivative bearing a fluorescein isothiocyanate (FITC) tag at the 9-position¹⁷⁸ and asialofetuin as the acceptor. This technique has the advantage of being applicable for various enzyme subtypes by using a versatile acceptor, however, it does require the non-trivial preparation of the FITC-labelled CMP-Neu5Ac donor. Kumagai *et al.* have reported a multi-enzyme system allowing the screening of potential inhibitors against a wide variety of glycosyltransferases including glucosyl-, galactosyl-, glucuronyl-, xylosyl-, fucosyl- and sialyltransferases.¹⁷⁹ Three kinase enzymes are used in order to provide glucose-6-phosphate (G6P) starting from the nucleoside monophosphate resulting from the glycosylation reaction. G6P dehydrogenase converts then resazurin, in a dose-dependant manner, to resorufin and the fluorescence of the solution is measured. This method presents the advantage of allowing HTS of compound libraries.

MS-based Assays: Hosoguchi *et al.* designed a MS-based protocol suitable for HTS of potential GT inhibitors. The authors monitored the inhibition rate by looking at the

amount of glycosylated acceptor using MALDI-TOFMS.¹⁸⁰ More recently, Preidl *et al.* designed a binding affinity assay in which the dissociation constant was determined by comparing the anisotropy changes in fluorescence polarisation.¹⁸¹ An alternative technique consists in immobilising the acceptor sugar on a plate or a cellulose membrane. This method was described using *N*-acetyllactosamine¹⁸² or trisialyl lactoside (GT3) by click-chemistry.¹⁸³ Other assays using multi-enzyme systems combined with spectrophotometry^{53,184} or colorimetry¹⁸⁵ have also been reported.

B. Cell-based Assays

Altered sialylation of the cell surface as a result of ST inhibition is expected to affect metastatic cell behaviours such as invasion, adhesion and migration, rather than cell viability. For this reason, classic cytotoxicity tests such as MTT and MTS assays have shown very little to no toxicity for a range of ST inhibitors, which greatly enhances their drug development potential.^{56,186-188} Thus, the most prevalent assays in this area are those that measure cell invasion,¹⁸⁹ cell migration using Transwell chamber migration assays,^{55,186} cell adhesion to plates coated in collagen IV, fibronectin or laminin,^{55,56,189,190} along with traditional wound healing assays.^{55,186,191} Further anti-metastasis assays include those that assess activation of integrins and downstream mediators of integrin signalling, such as FAK (focal adhesion kinase),^{56,186} and PI3K/Akt signalling via Western blot analysis.⁸⁷ Other assays looking at the functional consequences of altered cell surface sialylation include examining effects on selectin-mediated leukocyte rolling in HL-60 cells and MS analysis of the *N*-glycans from inhibitor-treated cells as described by Rillahan *et al.*,¹⁸⁷ as well as effects on NCAM polysialylation via ST8Sia inhibition.^{188,192} Table II shows some of the cancer cell lines used in cell-based evaluation of ST inhibition.

Other cell-based methods of determining the extent of ST inhibition include flow cytometry analysis of cell surface sialylation using biotinylated lectins. For example *M. amurensis* lectin II, which is specific for α -2,3-linked sialic acid and *Sambucus nigra* lectin, which is specific for α -2,6-linked sialic acid.^{55,56,181,187,189}

Lectin affinity has also been used to precipitate lectin/sialylated protein complexes out of cell lysates, followed by cleavage of the complex and Western blot analysis of the sialylated proteins.^{55,56} Cellular uptake of labelled ST inhibitors has been investigated using a range of cell staining techniques and confocal microscopy, successfully demonstrating the cell permeability of a range of ST inhibitors.^{56,181}

Table II. Ccell lines used in sialyltransferase inhibition studies.

Cancer type	ST type	Cell line	Analysis	Reference
Breast cancer	ST3/6Gal I	MCF-7 MDA-MB-231	Viability, adhesion, migration, invasion, flow cytometry, lectin affinity, FAK Cell proliferation, wound healing assay	Chang, 2011 ¹⁸⁵ Fu, 2015 ¹⁹⁰
Colon adenocarcinoma	indirect	NL-17	Cell attachment, cell-induced platelet aggregation	Kijima-Suda, 1988 ¹⁸⁹
	ST8Sia II & IV	HL-60	Immunoblotting, quantitative RT-PCR	Horstkorte, 2004 ¹⁹¹
Pheochromocytoma, Leukemia, Lymphoma	ST8Sia II & IV ST3Gal I ^a ST6Gal I	PC-12 HL-60, Ramos cells	Flow cytometry, glycan profiling, lectin affinity, leukocyte rolling assays	Rillahan, 2012 ¹⁸⁶
Lung cancer	ST3/6Gal I	CL1-0, CL1-5, CL1-5F4, A549, H1299, BES-6 ^b	Viability, adhesion, migration, flow cytometry, <i>in vivo</i> lung metastasis assay	Chiang, 2010 ⁵⁶ Chen, 2011 ⁵⁵
Melanoma	ST3Gal I	B16F10 (murine)	Adhesion, migration, invasion, flow cytometry, <i>in vivo</i> lung metastasis assay	Chang, 2006 ¹⁸⁸
Neuroblastoma	ST8Sia II	SH-SY5Y	Migration, flow cytometry, Western blot	Al-Saraireh, 2013 ¹⁸⁷
	ST3Gal II, ST6Gal I	B35, CHO	Flow cytometry, immunoblotting	Preidl, 2014 ¹⁸⁰

^a Also ST3Gal II, IV, VI.^b Normal cell line.

In vivo evaluation of ST inhibitors in a range of experimental animal xenograft models has been reported. These include inhibition of lung metastasis in BALB/c mice induced by injection with A549 human lung adenocarcinoma epithelial cells;⁵⁶ B16F10 human melanoma cells;¹⁸⁹ murine colon adenocarcinoma 26 sublines of high (NL-17) and low (NL-44) metastatic potential;¹⁹³ or highly metastatic murine 4T1-Luc breast cancer cells.⁵⁵ A multidrug resistant (MDR) leukemia xenograft model in mice injected with K562 (human erythromyeloblastoid leukemia) and K562/ADR (adriamycin-resistant) cells has been described by Zhang *et al.*⁸⁷ In earlier work, a liver metastasis assay was used to assess the role of sialylation in hepatic tumour growth in athymic male Swiss nude mice injected with human colorectal cells.¹⁹⁴ Zebrafish are an emerging live whole animal model for early pharmacological assessment of safety and toxicity in drug development,¹⁹⁵ and have been used for examining the expression and evolution of ST8Sia III¹⁹⁶ and its link to neuronal migration.¹⁹⁷ Mapping of the glycome and glycosyltransferases of zebrafish liver cells provides a useful tool for future glycosylation studies in this model system.¹⁹⁸

3. CRYSTALLOGRAPHIC DATA

As a result of the tremendous therapeutic potential associated with STs, the design and development of selective ST inhibitors is a promising new challenge. Efforts have been made to identify and understand the structural differences between the different ST subtypes.

Accordingly, the crystal structures are obtained with an additive co-crystallized with the enzyme, such as CMP (**5**), CDP (**6**), the inhibitor CMP-3F-Neu5Ac¹⁹⁹ (**7**) or several different sugars, including oligosaccharides. The presence of these compounds provides information about the location of the active site.

Bacteria were found to be a good source for STs. From *Campylobacter jejuni*, Cst-I was isolated and found to promote α -2,3-sialyl patterns²⁰⁰ and Cst-II, which is multifunctional, catalyses both α -2,3- and α -2,8-sialylation.²⁰¹ Crystallization of PM0188, an α -2,6-ST from *Pasteurella multocida*, has been reported by Kim *et al.*^{202,203} However, PM0188 presents very low sequence homology to other sialyltransferase families. From the same bacteria, Δ 24PmST1 was also examined. The enzyme shows α -2,3-ST, α -2,6-ST, sialidase and trans-sialidase activity.^{204,205} Additional mutation provided another enzyme, derived from PM0188, with inverted substrate selectivity, i.e. from α -2,3 to α -2,6. The crystal structure of this mutant enzyme P7H-M117A, expressed in *Pasteurella dagmatis* was reported by Schmöelzer *et al.*²⁰⁶ Ni and Sugiarto *et al.* successfully generated a mutant version of Δ 24PmST1 and described the crystal structure of the name D141N and M144D, respectively.²⁰⁷ M144D showed significantly decreased sialidase activity. In a similar approach, the crystal structure of the mutant bifunctional α -2,3-ST and α -2,6-ST Δ 29NST was obtained from the bacterias *Neisseria meningitides*. Δ 16psp26ST is a truncated form of JT-ISH-224, mono-functional β -galactoside α -2,6-ST, cloned from *Photobacterium phosphoreum* and its crystal structure was reported by Kakuta *et al.*²⁰⁸ Δ Npp23ST, also a truncated form of JT-ISH-224, was later crystallized by Iwatani.²⁰⁹ Huynh later reported the structures of Δ 15Pd2,6ST(N) and Δ 112Pd2,6ST(N) which also are α -2,6-STs from *Photobacterium damsela*.²¹⁰ The first mammalian ST crystal structure was reported by Rao *et al.*²¹¹ The crystalized enzyme is a porcine ST3Gal I variant and presents approximately 85% homology with the corresponding human ST. From this data, the authors later proposed a mode of binding for the donor CMP-Neu5Ac and the acceptor sugar.²¹² More recently, both rat²¹³ and human²¹⁴ ST6Gal I crystal structures were also reported. The latest human crystal structure reported is ST8Sia III (Fig. 4).²¹⁵

The crystallographic data provides insight on the mode of action of STs and allows the use of computational tools to assist structure-based design of inhibitors. Bacterial and mammalian STs crystal structures are summarised in table III. All reported PDB files are available on the RCSB Protein Data Bank website (www.rcsb.org).

Figure 4. Nucleotides binding to ST crystal structures. CMP **5** (A and B) and CDP **6** (C) are shown as a stick model. (A) The porcine ST3Gal I-CMP complex (PDB ID: 2WNB). (B) the human ST6Gal I-CMP complex (PDB ID: 4SJ2), and (C) the human ST8Sia III-CDP (PDB ID: 5BO6).

Collectively this data confirms the mechanism for the reaction, first proposed by Horenstein (Fig. 2).²¹⁸⁻²²² The reaction undergoes mechanistic pathways, which are very similar to other glycosyltransferases.²²³ The CMP fragment of the donor behaves as a leaving group. Catalytic residues are usually histidine or tyrosine, however arginine residues have also been observed in some cases. They play a key role in the deprotonation of the hydroxyl group of the acceptor sugar and in activating the phosphate group. Other residues in the active site help in positioning both donor and acceptor in their respective binding pockets.²⁴ The transition state proposed by Horenstein appears to be applicable for all ST subtypes. During the transfer, the acceptor approaches the anomeric centre of the sialic acid fragment by the side opposite to the CMP residue. At this stage, a planar oxocarboxenium intermediate takes place in an S_N2 fashion. The final products are CMP and the sialylated sugar (Fig. 2). This proposed mechanism was recently re-explored and assessed by Schmölzer *et al.*²²⁴ In addition, the crystallographic data complement previously described structural analysis studies on ST3Gal I,²²⁵ ST6Gal I,²²⁶ and polysialyltransferases.²²⁷

Table III. Examples of reported crystallographic data for ST subtypes and their corresponding PDB file names.

Table III. Examples of reported crystallographic data for ST subtypes and their corresponding PDB file names.

Enzyme	Organism	PDB file	Reference
Cst-I	<i>Campylobacter jejuni</i>	2P2V, ^a 2P56	Chiu, 2007 ¹⁹⁹
Cst-II	<i>Campylobacter jejuni</i>	1RO7 ^a , 1RO8 ^b	Chiu, 2004 ²⁰⁰
Cst-II	<i>Campylobacter jejuni</i>	2X61, ^{b,h} 2X62, ^b 2X63 ^b	Lee, 2011 ²¹⁵
PM0188	<i>Pasteurella multocida</i>	2IY7, ^a 2IY8, ^{a,c} 2C83, 2C84 ^b	Kim, 2006 ^{201 202}
Δ24PmST1	<i>Pasteurella multocida</i>	2EX0, 2EX1 ^b	Ni, 2006 ²⁰⁴
Δ24PmST1	<i>Pasteurella multocida</i>	2IHJ, ^a 2IHK, ^a 2IHZ, ^{a,c} 2ILV, ^{b,c}	Ni, 2007 ²⁰³
D141N	<i>Pasteurella multocida</i>	2IIB, ^b 2II6 ^b	Ni, 2006
M144D	<i>Pasteurella multocida</i>	3S44 ^b	Sugiarto, 2012 ²⁰⁶
P7H-M117A	<i>Pasteurella dagmatis</i>	4V2U, 4V38, 4V39, 4V3B, ^b 4V3C ^b	Schmölzer, 2015 ²⁰⁵
Δ29NST	<i>Neisseria meningitides</i>	2YK4, 2YK5, ^b 2YK6, ^d 2YK7 ^a	Lin, 2011 ²¹⁶
JT-ISH-224	<i>Photobacterium phosphoreum</i>	2Z4T ^{b,c}	Kakuta, 2008 ²⁰⁷
ΔNpp23ST	<i>Photobacterium phosphoreum</i>	2ZWI ^b	Iwatani, 2009 ²⁰⁸
Δ15Pd2,6ST(N)	<i>Photobacterium damsela</i>	4R83, 4R84 ^a	Huynh, 2014 ²⁰⁹
Δ112Pd2,6ST(N)	<i>Photobacterium damsela</i>	4R9V	Huynh, 2014 ²⁰⁹
ST3Gal I	<i>Sus scrofa</i> (pig)	2WML, 2WNB, ^{b,e} 2WNF ^e	Rao, 2009 ²¹⁰
ST6Gal I	<i>Rattus norvegicus</i> (rat)	4MPS	Meng, 2013 ²¹²
ST6Gal I	<i>Homo sapiens</i> (human)	4JS1, ^f 4JS2 ^b	Kuhn, 2013 ²¹³
ST8Sia III	<i>Homo sapiens</i> (human)	5BO6, ^d 5BO7, ^g 5BO8, 5BO9 ^{a,h}	Volkers, 2015 ²¹⁴

The crystal structure of the enzyme was obtained co-crystalized with a) CMP-3F-Neu5Ac **7**, b) CMP **5**, c) Sia6SLacNAc d) lactose, e) CDP **6**, f) 2-nitrophenyl-Galβ1,3GalNAc, g) Cytidine, and h) CTP.

4. SIALYLTRANSFERASE INHIBITORS

Promising *in vivo* results endow ST inhibitors with a considerable interest in their therapeutic potential, particularly in the area of cancer. A large number of compounds have been described to date. They belong to different classes of molecules and scaffolds and recent discoveries based on natural products or high throughput screening provide even more molecular diversity. Consequently, drawing a structure-activity relationship model is a difficult task.

A. Inhibitors from Nature

Although natural products were used for thousands of years, their isolation and application still play critical roles in modern drug development, especially for antibacterial and antitumour agents. Soyasaponin I (**8**) from soybean is a rigid pentacyclic system with a trisaccharide component that showed significant inhibition of ST3Gal *in vivo* ($K_i = 2.1 \mu\text{M}$) (Fig. 5). Along with good cell permeability, this compound exhibited properties relating to invasive/metastatic behaviours of cancer cell lines.²²⁸ Soyasaponin I was also found to specifically inhibit the expression of α -2,3-linked sialic acids without affecting other glycans on murine melanoma cells.¹⁸⁹ Various steroidal compounds have been reported to exert inhibitory effects on ST3Gal I among them, epiandrosterone succinyl ester, isolated from the

parasite *Schistosoma japonica* and known as a glutathione *S*-transferase inhibitor,²²⁹ had an IC_{50} value of 350 μ M.¹⁸⁹ From this example, efforts to provide new ST3Gal I inhibitors based on aminoacids esters (IC_{50} = 480 μ M) or phosphate (IC_{50} = 400 μ M) conjugates of epiandrosterone were undertaken, however the compounds showed lower potency than the parent compound.²³⁰

Another steroidal derivative that has attracted attention is lithocholic acid (**9**) (Fig. 5). This compound has been shown to inhibit ST3Gal I (IC_{50} = 21 μ M). Consequently, a series of lithocholic acid derivatives were designed by Chang *et al.* and their activities evaluated on rat ST3Gal,²³⁰ wherein, succinic and aspartic acids conjugates gave a similar range of affinity with the enzyme. On the other hand, the reduction of the carboxylic acid group to the corresponding alcohol significantly decreased activity (IC_{50} = 351 μ M). The same effect was observed for its phosphorylated analogue (IC_{50} > 100 μ M). The oxidation of the secondary alcohol to a ketone also decreased the activity (IC_{50} = 139 μ M). Nevertheless, the conversion of the primary alcohol derivative to the azide counterpart gave way to a series of 1,4-disubstituted triazoles, conveniently obtained by click chemistry. In general, compounds bearing an acidic carboxylic group attached to the side chain generally presented increased activity when compared to the analogous alcohol. The most active lithocholic acid derivative, the L-Asp compound (**10**) (Fig. 5), was the object of further biological evaluation. The so-called Lith-*O*-Asp derivative was found to inhibit migration and invasion in various cancer cell lines and showed inhibitory activity towards human umbilical vein endothelial cells.⁵⁵ By decreasing the sialylation of integrin- β 1 and consequently inhibiting the expression of phospho-FAK, phosphopaxillin and matrix metalloproteases, Lith-*O*-Asp suppressed cancer cell metastasis. This compound was later the object of further clinical studies which suggest a promising potential for metastasis treatment.

Other lithocholic acid derivatives showed encouraging results from pre-clinical experiments. AL-10 (**11**) (Fig. 5), which is structurally related to Lith-*O*-Asp, exhibited inhibition of ST3Gal I (IC_{50} = 0.88 μ M) and ST6Gal I (IC_{50} = 1.50 μ M) in enzyme inhibition assays.⁵⁶ As observed with Lith-*O*-Asp, the sialylation of α 5, α v and β 1 integrins was repressed. As a result, adhesiveness to fibronectin and laminin was reduced, leading to decreased migratory activity of the metastatic cells. The test of the antimetastatic effect of AL-10 *in vivo* revealed a 40% reduction of lung metastasis in serum biochemical assays. More recently, further derivatives have been prepared based on the encouraging results observed with the Lith-*O*-amino acid compounds.¹⁹¹ Therefore, short peptidic units were attached to the 3-*O*-position of the steroid moiety. All these

compounds were tested against ST3Gal I and some of the most potent were also evaluated against ST6Gal I. Most compounds showed moderate activity against the enzymes in the low micromolar range (**12-16**) and inhibited the migration of highly metastatic cancer cell lines (MDA-MB-231) (Fig. 5).

Some examples of lithocholic acid derivatives exhibiting ST inhibition are commercially available, such as lithocholic acid (CAS: 434-13-9), lithocholylglycine (CAS: 474-74-8) and lithocholyltaurine (CAS: 516-90-5).

However, the binding site for the lithocholic acid derivative is not clearly defined and could possibly be allosteric, these compounds shown to exhibit a wide range of off-target activities including inhibition of the nuclear pregnane X receptor,²³¹ antagonism of the Eph-ephrin system,^{232,233} activation of Vitamin D receptor,²³⁴ agonism of the TGR5 receptor,²³⁵ interaction with acetylcholine receptors²³⁶ and increased invasiveness by up-regulating urokinase-type plasminogen activator receptor in colon cancer.²³⁷

Three metabolites isolated from the fungus *Stachybotrys cylindrospora* have been reported as ST inhibitors. Lin *et al.*, evaluated the activity of these spirocyclic drimanes (Mer-NF5003F or stachybotrydial (**17**), and the lactone counterpart (**18**) towards ST3Gal I, ST3Gal III and ST6Gal I using a radiolabelled assay¹⁶² as well as some fucosyltransferases and galactosyltransferases (Fig. 6).²³⁸ All compounds showed affinity against the enzyme in the low micromolar range as well as some selectivity for ST6Gal I. These terpenes were also shown to exhibit a range of off-target effects including *myo*-inositol monophosphatase inhibition,²³⁹ activation of Glu-plasminogen,²⁴⁰ antiviral and antiplasmodial²⁴¹ as well as pancreatic cholesterol esterase inhibition.²⁴²

Marine metabolites currently constitute an attractive source of new bioactive structures in medicinal chemistry. The Taiwanese cyanobacteria *Lyngbya Majuscula* was found to produce two fatty amides, isomalyngamide A and A-1 (**19**), which share a common 4-methoxypyrrol-2(5*H*)-one scaffold (Fig. 5). The activity of these compounds was examined by Chang *et al.* against proliferation and migration of metastatic human breast cancer cells (MCF-7 and MDA-MB-231).¹⁸⁶ The study included ST3Gal I inhibition measurement of the natural products and their synthetic analogues using Schmidt's HPLC-based assay.¹³⁵

Isomalyngamides themselves show high inhibitory activity ($IC_{50} = 77.2 \mu M$ and $65.7 \mu M$ respectively) and the synthetic analogues (**20**) displayed even greater affinity (Fig. 5). Once again, the authors suggest that the hypersialylation of $\beta 1$ -integrin may play a key role in cancer migration processes.

Figure 5. Sialyltransferase inhibitors from Nature.

Several flavonoids exhibiting inhibition of STs have been recently reported by Hidari *et al.*²⁴³ The radiolabelled assays,^{172,173} performed using both rat, human ST6Gal I and rat ST3Gal I, revealed potent inhibitory activity for some of these compounds, while hydrogenation of the pyranone ring led to loss of the activity. Successful inhibition was obtained with derivatives bearing a 3',4',5'-trihydroxyphenyl moiety (**21-23**) (Fig. 5). The authors also suggest that glucosylation at the 6-position of the chromene ring may affect specific-linkage inhibition of sialylation. Kinetic properties of these compounds were also examined for ST6Gal I and revealed mixed inhibition profiles. Epigallocatechin gallate (**24**), was previously shown to moderately inhibit rat brain ST3Gal I (EC 2.4.99.4), $IC_{50} = 2.7 \mu M$.²⁴⁴ This compound also inhibits fucosyltransferases in a similar order of potency.

B. Inhibitors Discovered from High Throughput Screening

Paulson *et al.* discovered new small molecules with high ST inhibitory activity using high-throughput screening (HTS) of compound libraries.¹⁷⁷ The molecules were evaluated against porcine ST3Gal I, rat ST3Gal III, human ST6Gal I as well as human FUT6 and human FUT7. This study includes mildly to very selective inhibitors (**25-26**) of ST3Gal III with high affinity for the enzyme ($IC_{50} = 1.7 \mu M$) whereas the activity towards the other enzymes ST3Gal I, ST6Gal I, FUT6 and FUT7 was negligible (Fig. 6). In addition, this compound was found to be competitive with the natural donor substrate site.

High throughput screening of synthetic combinatorial libraries identified a new hexapeptide inhibitor for ST3Gal I.²⁴⁵ Comparison of 114 different peptides ($NH_2-X_6X_5X_4X_3X_2X_1$) revealed that the terminal tryptophan residue (X_1) was optimal, and glycine, asparagine and arginine were less active. Tryptophan also appeared to be ideal at the three following positions (X_2 , X_3 , and X_4). The authors determined a K_i value of $8.8 \mu M$ for hexapeptide NH_2 -GNWWWW (**27**), showing the most promising activity (Fig. 6). Kinetic studies revealed this peptide to be a noncompetitive inhibitor of ST3Gal I. This compound also exhibits inhibitory activity toward ST6GalNAc I and ST6GalNAc III.

Figure 6. Sialyltransferase inhibitors discovered by high throughput screening.

Not only organic molecules can afford biological activity, but some polyoxomolybdates have also been indentified as antitumour,²⁴⁶⁻²⁴⁸ antibacterial²⁴⁹ or antiviral agents.²⁵⁰ In order to elucidate the mechanism of action of such complexes, Seko *et al.* explored the inhibitory activity and selectivity of molybdate, tungstate and vanadate compounds towards a few glycosyltransferases including mutated ST3Gal I and ST3Gal II.²⁵¹ IC₅₀ values of the tungstates for ST3Gal I are potent (0.1-1 nM), ten fold lower than for ST3Gal III, ST6Gal I and ST6GalNAc I. The other enzymes evaluated (β4Gal-T1, β3Gn-T2 and FUT3) were inhibited only weakly or not at all. The vanadate-type compounds appeared to be more specific towards ST subtypes than the tungsten-type derivatives.

C. Acceptor Analogue Inhibitors

Since the early modified disaccharides reported by Hashimoto's group,²⁵² a number of different types of acceptor-analogue have been designed. *N*-acetyl-β-lactosamide has been modified at the 6-position of the galactose residue, including deoxy (**28**), mercapto (**29**), *O*-THP (**30**) and disulfide (**31**) groups. These compounds afforded modest inhibitory activity against rat ST6Gal I (K_i = 0.76 to 4.14 mM) and revealed that the 6'-OH group is not essential for substrate binding.²⁵³ Okazaki *et al.* also reported methyl 5a'-carba-β-lactoside derivatives (**32-34**) and tested them against rat ST3Gal I and ST6Gal I.²⁵⁴ The inhibitors display some activity in the μM range and up to 1/2.8 selectivity ratio was observed. These inhibitors exhibit only modest activity (Fig. 7). To our knowledge, no acceptor-analogue

inhibitors were reported recently and the existing compounds have been previously reviewed.¹⁴²

Figure 7. Sialyltransferase inhibitors based on acceptor analogues.

D. Cytidine-based Inhibitors

The structure of CMP-Neu5Ac **4** can be divided into three major fragments: the nucleoside fragment, the sialic acid fragment and the phosphodiester linker.

Cytidine fragment: Over the last decade, several research groups focused on the design of donor analogue inhibitors. Prevention of glycosyl transfer is mediated by binding in the active site of the enzyme in competition with the natural glycosyl donor CMP-Neu5Ac (**4**). These inhibitors generally contain a cytidine component which was found to be essential for binding to the enzyme.²⁵⁵ CMP **5**, CDP **6** and CTP were shown to exhibit inhibition (K_i = 16 to 50 μ M against rat liver ST6Gal I, K_i = 10 μ M competitive inhibition against ST8Sia II).¹⁸⁸ Uracil and resorcinol derivatives show reduced affinity for ST6Gals²⁵⁵ while being non-competitive.²⁵⁶ Adenine derivatives showed very low if any activity against ST3Gal I²⁵⁷ and ST6Gal I.²⁵⁶ 5-Methylcytosine derivatives showed selectivity for ST8Sia²⁵⁸ and the 5-FU analogue, for which inhibitory activity was twice as potent.²⁵⁹

Substitutions and alterations of the ribose fragment were also explored and revealed some differences between the ST subtypes. 2'-O-Methyl-CMP promoted 84% inhibition at 250 μ M against ST3Gal-III and no inhibition against ST6Gal I (CMP exhibited 67% and 49% inhibition respectively at the same concentration).²⁵⁸ This result suggests that the environment

in the active site is significantly different from one enzyme subtype to another. In addition, this confirms that the empty pocket neighbouring the 2'-hydroxyl group, as observed on the pST3Gal I crystal structures (PDB ID: 2WNB), provides an avenue for selective ligands. 2'-Deoxycytidine was evaluated against human serum ST activity and showed very mild inhibition, ranging from 5.9% at 100 μ M to 32.5% at 1 mM.²⁵⁶ Ara-CMP and ara-CTP exhibited mild competitive inhibition against ST6Gal I (K_i = 1.10 mM and 0.50 mM respectively).²⁵⁶ Replacement of the furan ring by a 1,3-dioxolane moiety gave inactive compounds (IC_{50} > 100 μ M).²⁵⁷ In a different approach, Tanaka *et al.* explored the replacement of the entire ribose moiety by a peptidic scaffold.²⁶⁰ The general outcome of these studies led to the consideration of cytidine as essential for the activity on ST6Gal I.²⁵⁵ Most cytidine nucleotides act as competitive ST6Gal inhibitors.²⁵⁶ Other examples bearing a 5FU moiety exhibit modest activity such as the sialic acid-nucleoside conjugate KI-8110 (**35**) (Fig. 8).²⁶¹ This compound was preliminarily considered a ST inhibitor by indirect evidence, observing diminution of sialylation of the cell surface of treated cells. However, it was later found to inhibit CMP-Neu5Ac transport into the Golgi.²⁶²

Linker: An interesting feature in KI-8110 **35** is the fact that the compound does not bear any group mimicking the native phosphate linker. The phosphate linker between the cytidine and sialic acid fragments appears to contribute largely to the binding to the active site of the enzyme. A few isosteric replacements for the phosphate group were investigated, including phosphoramidate analogues prepared stereoselectively.²⁶³ The resulting inhibitors exhibited moderate activity in the low micromolar range. Replacement of the native phosphate linker by phosphonate esters was also evaluated. When the phosphonate group is directly linked to the anomeric centre of the sialic acid fragment, weak inhibition is observed (K_i = 250-780 μ M against rat liver ST6Gal I).¹³⁷ However moderate to weak activity is observed when tethered via an ethylene linker (IC_{50} = 47-3300 μ M against rat ST3Gal I and 340-4300 μ M against rat ST6Gal I).²⁶⁴ By using simplified molecular tools, Lee *et al.* prepared a series of 5'-triazolonucleosides to evaluate their activity on rat liver α -2,3-ST.²⁵⁷ In agreement with previous observations, the adenine derivatives failed to prove significant inhibition. Among the cytidine based molecules, only compound **36** showed significant activity (IC_{50} = 37.5 μ M). Interestingly, the presence of a phenyl group seems to be a determinant factor, presenting a 2-fold improvement over the cyclopentyl counterpart **37** (44% inhibition at 300 μ M) (Fig 8). In order to increase the hydrophobicity of the compounds and improve their cell permeability, Kumar *et al.* explored CMP-Neu5Ac analogues bearing non-charged linkers instead of the phosphate group.²⁶⁵ Three neutral patterns were explored: triazole (**38**),

sulphonamide and amide. Further modifications on the sialyl moiety also included 2-deoxy-2,3-dehydro-acetylneuraminic acid derivatives or aromatic rings. The inhibitory activity of these compounds was evaluated against the bacteria *C. jejuni* Cst 06, using a HPLC-based technique,¹³⁵ and relatively low inhibition observed (10.7-79.4% at 500 μ M). The 5'-triazole nucleoside analogs, obtained by click chemistry, appeared to be competitive inhibitors. On the other hand, two of the most active compounds (**39-40**) showed non-competitive inhibition, suggesting the nitro group could play a role in the binding with the active site of the enzyme (Fig. 8).

Figure 8. Structure of CMP-Neu5Ac derivatives with a modified linker.

Sugar fragment: The most efficient modifications appear on the sialyl residue. The non-sialyl framework CMP-quinic acid derivative, for example, displays practically identical K_i values (44 μ M against rat liver α -2,6-ST) to CMP-Neu5Ac **4** (K_i = 46 μ M against rat liver α -2,6-ST). For this purpose, the use of sialyl mimetics, which do not possess glycosidic bonds, are suitable to prevent glycosyl transfer. Corresponding phosphonates (sialic acid mimicked by a phosphonate or carboxylate linked via a non-hydrolysable spacer) were less potent inhibitors. Phosphoramidates containing amino acid as sialyl mimetics (R-CH(CO₂H)NH-CMP) were also found to possess low inhibition towards ST3Gal and ST6Gal. Based on this

knowledge, Schmidt's group prepared a number of CMP-Neu5Ac analogues including cyclohexanone moieties, acetylaminocyclohexane and their hydroxylated derivatives.²⁶⁶

Inspired by 3-fluorosialic acid as a neuraminidase inhibitor, Burkart *et al.* prepared CMP-3F-Neu5Ac (**7**) *via* the phosphoramidate route.¹⁹⁹ The same compound was also synthesised using a chemoenzymatic strategy.²¹⁶ CMP-3F-Neu5Ac inhibits ST6Gal I with $K_i = 5.7 \mu\text{M}$ and became a reference compound for inhibition assays. It was also co-crystalised with various ST subtype, enabling identification of the active site (cf section 3).

In analogy to some inhibitors mimicking the transition-state bearing a bicyclo[3.1.0]hex-2-ene moiety firstly described by Sun,²⁶⁷ Niwayama *et al.* developed diacid derivatives.²⁶⁸ The intention of such compounds undergoes a Meinwald rearrangement and the stereospecificity is controlled by the use of pig liver esterase. The study provided four new compounds, however assessment of their biological activity has not been reported.

Click chemistry is an elegant way to obtain libraries of highly functionalized compounds. Using this methodology, Hosoguchi *et al.* reported a library of over 70 donor-based inhibitors obtained by click reactions between 5'-azidocytidine and 36 commercially available alkynes.¹⁸⁰ The library is comprised of CMP-Neu5Ac analogues bearing a functionalized triazole group at the 5 or 9-position of the sialyl moiety, respectively, assuming that these modifications should not significantly affect the binding of the compounds in the active site of the enzyme. The ST inhibition was determined using a MALDI-TOF MS-based HTS towards ST3Gal III, ST6Gal I as well as α -1,6-FucT. These molecules display inhibitory profiles toward the considered enzyme subtypes, ranging from 15 to 80 %, and even selectivity, providing important information about the donor binding sites. The authors suggest that a flexible loop in mammalian ST6Gal I might be different from those of ST3Gal III, even if both share the same natural donor. The derivatives of the 9-modified library did not show significant inhibitory activity, but were recognized as the substrate by the enzymes. Compound **41** of the 5-modified library show potent inhibitory activity, whereby the compound showed a significant selectivity, with strong non-competitive inhibition of ST3Gal III ($\text{IC}_{50} = 8.2 \mu\text{M}$) over moderate competitive inhibition of ST6Gal I ($\text{IC}_{50} = 125 \mu\text{M}$) (Fig. 9). This result clearly demonstrates that the sugar transfer reaction is not disturbed by bulky substitutions at the 5- or 9-position. It is also interesting to note that the most active compounds in this library bear a steroidal moiety.

In continuation of previous work initiated by Schmidt's group,¹³⁵ extended libraries of compounds have been synthesized providing active molecules at the submicromolar level on

rST6Gal I. Among them, the first series was obtained by coupling cytidine with functionalized α -hydroxyphosphonates, using the phosphoramidite route, activated with 1*H*-tetrazole.²⁶⁶ The α -hydroxyphosphonate portion of the molecule bears a substituted benzene ring, naphthalene or heterocycle²⁶⁹ and this building block was prepared using an asymmetric synthetic pathway to promote optically pure synthon. This study indicates that the configuration of the stereogenic centre attached to the phosphonate group has little influence on the activity. On the other hand, no relevant conclusion evolves from the electronic influence (donating or withdrawing) of the substituents present on the aromatic ring. However, compound **42**, bearing a 3-phenoxy group, presents a significantly increased affinity with the enzyme (Fig. 9). This relevant enhancement of the affinity implies the presence of aromatic aminoacids in the active site and the phenoxy group would thus promote an aromatic-aromatic interaction with them. Phosphoramidate analogues have been prepared stereoselectively,²⁶³ with the (*R*) compound found to exhibit competitive inhibitory activity ($K_i = 68 \pm 24 \mu\text{M}$) however less potent than their phosphodiester counterpart.

Building on the potent scaffold **42**, fluorescent probes were designed.¹⁸¹ Binding affinity of the compounds were evaluated using a fluorescence polarisation assay against rST3Gal II, hST6Gal I and bacterial PmST1 and Pd2,6ST(N). The probes were found to exhibit activity in the low nanomolar range. A fluorescein based tag was attached to the 3- or 4-position of the aromatic ring (**43**) using an acetamide linker and the corresponding analogues bearing a glycine spacer were also prepared (Fig. 14). The use of cell-based assays shed light on the permeability of the probe and provided evidence that the compounds reached locations inside the cell surrounding the nuclei and possibly to the trans-Golgi network. Other assays assessed the inhibition of sialylation in two cell lines, Chinese Hamster Ovary cells and B35 neuroblastoma cells. The authors suggest that a putative membrane receptor may play a key role in the vesicular uptake of the compounds.

Li *et al.* reported potent cyclopentane-based CMP-Neu5Ac analogues.²⁷⁰ These transition state inhibitors also bear a CMP fragment and an α -hydroxyphosphonate moiety and the sialic acid fragment was replaced by a five membered ring. In agreement with other inhibitors described above, the non-substituted cyclopentyl counterpart **44** displays weaker activity than the corresponding phenyl analogue. The design of more elaborate sugar mimic fragments was inspired by the structurally related sialidase inhibitor peramivir, which provided the most potent examples **45-47** (Fig. 9).

Figure 9. Sialyltransferase inhibitors bearing a modified sialic acid fragment.

E. Bisubstrate Analogues

Bisubstrate analogues constitute an interesting rational design approach for enzyme inhibition. Considering that the major difference between ST subtypes occurs primarily in the acceptor site, two main strategies have been explored for the design of bisubstrate analogues. These include a lactose unit tethered to an entirely intact CMP-Neu5Ac, or a CMP-KDN, moiety at the 3-axial position of the sialic acid residue.²⁷¹ Thioethers and alkanedithiols (**48**) were used as a linker to attach both fragments together (Fig. 10). All compounds were tested against rat ST3Gal I and ST6Gal I. The Neu5Ac derivatives were consistently more potent than the corresponding KDN counterparts and all of them exhibited modest inhibition in the low micromolar range.

Furthermore, a structure-activity relationship study also evaluated a bisubstrate analogue, prepared by Izumi's group and showed only weak inhibitory activity against recombinant rat ST3Gal I ($IC_{50} = 1.3$ mM) and ST6Gal I ($IC_{50} = 2.4$ mM, **49**, Fig. 10).²⁶⁴ This series present two key structural features. The linker was replaced by a phosphonate group which is no longer a leaving group and the donor and acceptor fragments are tethered with a phosphodiester moiety. The potential of bisubstrate ST inhibitors remains to be realised.

Figure 10. Structures of bisubstrate sialyltransferase inhibitors.

F. Metabolic Inhibitors

Paulson's group created two fluorinated monosaccharide analogues as metabolic ST inhibitors.¹⁸⁷ The strategy uses a modified bioprecursor of CMP-Neu5Ac to obtain the inhibitor inside the cell, synthesised *in situ* by the locally available enzymatic artillery, as previously observed with other glycosyltransferases.²⁷² For this purpose, peracetylated 3-fluorosialic acids (both separated diastereoisomers **50** and **51**) were able to cross the cell membrane and, after 'deprotection' by cytosolic esterases, get through salvage pathways to provide the potent donor-analogous inhibitor CMP-3F_{ax}-Neu5Ac (**7**) ($K_i = 5.7$ μ M), first described by Burkart¹⁹⁹ or the corresponding equatorial counterpart **52** (Fig. 11). The presence of a fluorine atom at the 3-position of the sialic acid prevents turnover by inductively destabilizing the oxocarbenium ion-like transition state of the catalytic process.²⁷³ Furthermore, the structural similarity of these compounds with the natural donors shuts down the *de novo* synthesis of CMP-Neu5Ac because of feedback loops in these pathways, induced by their accumulation. As long as evidence for Golgi localization of CMP-3F-Neu5Ac is not

clearly established, the inhibition of sialylation is thus thought to result from substrate depletion.

Although highly effective *in vitro*, subsequent *in vivo* studies by Paulson involving systemic blockade of sialylation in mice revealed the limitations of using global sialylation inhibitors such as P-3F_{ax}-NeuAc *in vivo* due to renal failure. Given the crucial role of sialylation in such a myriad of biological processes and particular the high level of expression in liver and kidney tissue, this is not unexpected. However, as stated by the authors, these results highlight the importance of developing selective, small molecule ST inhibitors for therapeutic purposes, while metabolic inhibitors will still have a crucial role to play as biological tools in uncovering more about the role of sialylation in disease progression.²⁷⁴

Figure 11. Hypothetical pathway for the activation of metabolic sialyltransferase inhibitors. *Conditions:* a) cytosolic esterases, b) salvage pathway.

In a similar approach, Horstkorte *et al.* explored the activity of 5-*N*-acyl-modified sialic acids on polysialyltransferases.¹⁹² The CHO-mutant 2A10 cells either ST8Sia II or ST8Sia IV were fed with 5-*N*-acyl-D-mannosamines. The results showed that the artificial sialic acid precursors bearing longer chains, i.e. propanoyl (**53**), butanoyl (**54**) and pentanoyl (**55**), at the

5-position considerably reduced cell surface α -2,8-sialylation (Fig. 11). Real time RP-PCR demonstrated that these compounds also affected gene expression. Unnatural sialic residues were also found to be incorporated into sialoglycoconjugates. These results suggest the formation of the corresponding CMP-Neu5Ac derivatives (**56-58**) which were prepared and tested in a different study²⁷⁵ with consistent findings.

5. STRUCTURE-BASED DESIGN

A. Computational Tools and Docking

Computational tools constitute an attractive avenue for the design of new selective and potent inhibitors. The recent characterisation of mammalian ST crystal structures have enabled visualisation of the substrate-enzyme interactions highlighted by previous mutational studies. As predicted, the donor binding site which is highly conserved between the different subtypes, remains consistent while the major variations occur in the acceptor site (Fig. 12A and B). Insight from crystal structures, such as rat and human ST6Gal I show many similarities. Inhibitions in rat enzymes are thus likely to be translatable into human with acceptable error aligning with biological results.

Using recent crystallographic data, research groups have started optimising new inhibitors by structure-based design. Kumar *et al.* designed non-charged inhibitors and docked them in the bacterial Cst-I enzyme (PDB: 2P2V).²⁶⁵ Chen *et al.* proposed a possible binding mode for the steroidal inhibitor Lith-*O*-Asp (**10**) in pST3Gal I.⁵⁵ Preidl *et al.* evaluated the binding site of potent fluorescent probes by docking in the bacterial PmST1 (PDB: 2IHZ) and pST3Gal I (2WNB).¹⁸¹ Montgomery *et al.* explored a range of donor and transition state analogues towards the human ST6Gal I crystal structure. Molecular dynamics was performed in order to validate the proposed mode of binding. Docking studies also reveal that compounds considered as donor/transition state analogues, including **42**, may in fact bind to the acceptor site (Fig. 12C).²⁷⁶ Sujatha *et al.* established an homology model for the six human ST3Gal I-VI by aligning their sequence with the bacterial enzyme Cst-II.²⁷⁷ Grewal *et al.* later created homology models for the human ST6GalNAc I, ST3Gal III and ST8Sia I using the crystal structure of the porcine ST3Gal I as a template and evaluated potential binding sites for the natural products curcumin and resveratrol.²⁷⁸

Docking of the inhibitor **41** described by Hosogushi *et al.*¹⁸⁰ into the pST3Gal I crystal structure shows a possible binding mode that could explain its selectivity and potency. The

steroidal moiety appears to fit in the acceptor site while engaging H-bonds with Thr²¹⁵ and Arg²⁶⁸ (Fig. 12D).

Figure 12. (A) CMP-Neu5Ac **4** (green sticks) and acceptor sugar (orange sticks) in hST6Gal I (PDB ID: 4JS2), and in (B) pST3Gal I (PDB ID: 2WNB), (C) the potent phenoxy derivative **42** (R) docked in the active site of rST6Gal I (PDB ID: 4MPS) and (D) the steroidal CMP-Neu5Ac derivative **41** docked in the active site of pST3Gal I (PDB ID: 2WNB)

B. Structure-Activity Relationship Model

As described in the previous section, ST inhibitors display a large molecular diversity. As a result, it is challenging to establish a SAR model. Donor- and transition-state analogue inhibitors are some of the most studied compounds and they have also exhibited the best activity. For these reasons, cytidine-based inhibitors will be the focus here (Table IV). Wang *et al.* elaborated a predictive 3D-QSAR model for α -2,6-ST based on comparative molecular field analysis. The authors provided a pharmacophore model of cytidine-based transition-state inhibitors.²⁷⁹ Jeanneau *et al.* described a structure-function analysis of hST3Gal I.²²⁵ Peptide sequence similarities have been examined by mutational analysis and three aminoacid residues

(two histidine and a tyrosine residues) were found to be essential for the catalytic activity. By using spin-labelled ligands, Liu *et al.* used an NMR study to identify the relative placement of the donor and acceptor sites.²²⁶ These methods constituted good complements or even alternatives to X-ray crystallography as most reported crystal structures were bacterial STs and the first examples of mammalian STs were described only recently (cf Section 3).

Table IV. Structure-activity summary for cytidine-based ST inhibitors discussed in this review.

	Sugar mimic	Linker	Nucleoside
ST3Gal		Triazole tolerated	Substitution tolerated at 2'-position Adenine not active
ST6Gal	<i>X</i> O >> NH > CH ₂ <i>Y</i> PO(OH) ₂ > CO ₂ H > OH > H <i>Z</i> O preferred on sugar-based derivative <i>R₁</i> PhO > HO(CH ₂) ₂ O > glycerol > 4-NO ₂ -C ₆ H ₄ -O <i>R₂</i> -NHAc > -OH > =O <i>R₃</i> OH preferred on sugar-based derivative	Phosphate optimal	Cytidine preferred 5-FU analogues tolerated Adenine, Resorcinol not active
ST8Sia	<i>R₂</i> Elongated acyl chain		Substitution tolerated at 2'- and 5-position

Comparing the inhibition data from the most active donor and transition-state analogue ST inhibitors, the following SAR picture emerges for this class of inhibitor. The cytidine moiety appears to play a key role in the interaction of ST inhibitors with the active site of ST6Gal I. Any small change dramatically decreases the affinity. The linker between cytidine and the sialic acid analogue is also of a great importance. A phosphate group, which acts as a leaving group in the natural donor CMP-Neu5Ac, provides the optimal activity. Many other functional groups have been evaluated, including amides, amino acids, sulfamides, triazole, phosphoramidate, phosphonate or even alkyl groups, however, all of these have afforded inhibitors exhibiting lower potency. The presence of a negative charge or an acidic group in the inhibitor appears to be favourable by interacting with the catalytic amino acid residues, histidine or arginine, in the active site of the enzyme. This is particularly the case at position Y, which replaces the carboxyl group of sialic acid.

The configuration of the carbon atom linking X and Y can be either *sp*² or *sp*³, and in several examples this position is a chiral centre. *R* or *E* configurations tend to be favoured at

this centre, however, this is also dependent on the substituents attached. The left hand part of the model represents the sialic acid mimic. The best results obtained to-date have been using either a sugar derivative, *e.g.* pyrane ring, or by replacing the complex highly functionalised sialic acid ring by an aromatic ring. The presence of a heteroatom at Z, to mimic the pyranyl oxygen atom of the natural donor, is also favourable. An oxygen atom at this position results in greater affinity than a nitrogen atom, which is greater than a carbon atom, suggesting a possible H-bond interaction with a polar residue in the active site.

R₁ refers to the polyhydroxylated side chain of sialic acid. A phenoxy group at R₁ has so far provided the greatest affinity, offering easier and cheaper synthetic pathways than a sugar related chiral polyol. The various ST crystal structures often reveal the presence of several hydrophobic residues in this location of the active site, suggesting that aromatic stacking interactions may occur between the phenyl group and a tryptophan or tyrosine residue. The selectivity achieved between the different enzyme subtypes in recent reports indicates that positions R₂ and R₃ could be interesting tools in the future for developing new selective inhibitors.

6. COMPOUNDS WITH INDIRECT ACTIVITY ON SIALYLTRANSFERASES

In the T84 colon cancer cell line, the secondary bile acid, deoxycholate (DOC, **59**) and the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA, **60**) were found to decrease expression of ST6Gal I to 30% and 13% of the control respectively, mediated by Ca²⁺ and protein kinase C (Fig. 13).²⁸⁰ Similar results were previously observed while examining the influence of sodium butyrate on MUC1 expressing Panc1 pancreatic cancer cell line.²⁸¹ Gene expression was monitored by real time PCR for ST3Gal III, ST3Gal IV, ST3Gal VI as well as some glycosyltransferases and β -actin.

Triptolide (**61**) is an immunosuppressive and antiinflammatory diterpenoid epoxide isolated from the perennial vine *Tripterygium wilfordii* (thunder god vine) native to China, Japan and Korea (Fig. 13).^{282,283} The vine has been used in Traditional Chinese Medicine for centuries for treating inflammation, in particular rheumatoid arthritis, and autoimmune diseases. Triptolide, one of the major active constituents of the vine, has been shown to inhibit cell proliferation in leukemia, neuroblastoma, pancreatic, ovarian, gastric and lung cancer cell lines.^{284,285} Various modes of action have been ascribed to triptolide including its ability to cause apoptosis via inhibition of HSP70^{286,287} and by blocking TNF- α -induced activation of NF- κ B.²⁸⁸ Interestingly, triptolide has also been found to downregulate human GD3

synthase (hST8Sia I) gene expression in SK-MEL-2 human melanoma cells.^{289,290} Triptolide has reduced growth and metastasis of tumours in various rodent models including polycystic kidney disease,²⁹¹ neuroblastoma, melanoma, and pancreatic, breast and gastric cancers, including S2-013, one of the most aggressive pancreatic-tumour derived cell lines.^{292,293} The antitumour effect of triptolide was found to be comparable or superior to a range of conventional antitumour drugs including Taxol, doxorubicin, and cisplatin, as well as retaining activity towards drug-resistance cell lines in cell culture studies.²⁹³ In addition to its antitumour activity, triptolide **61** has multiple reported functions, including immunosuppressive, antiinflammatory, and antifertility activity, and is also emerging as a promising new candidate for Alzheimer's disease based on its ability to inhibit BACE1 in a transgenic mouse model.²⁹⁴ Accordingly, there are several synthetic studies on triptolide and its analogues since its first report.²⁹⁵⁻²⁹⁹ Although its physical properties have limited triptolide's therapeutic potential, a synthetic prodrug analogue, minnelide (**62**), is showing equal promise including against tumour-initiating cells responsible for treatment relapse in pancreatic cancer, with minnelide currently in Phase I clinical trials for pancreatic and advanced gastrointestinal cancers at the time of writing (Fig. 13).³⁰⁰⁻³⁰⁵

Figure 13. Natural product derived and synthetic analogues with indirect inhibitory activity on STs.

Although there is only one study reporting the ST inhibitory activity of triptolide in melanoma, the diverse range of functions of this compound and potent activity towards high ST-expressing cancers such as pancreatic and ovarian cancer (see Fig. 4), including their drug-

resistant cell lines, raise the possibility that disruption of cell surface sialylation may play a key role in this compound's activity in a wide range of diseases.

Sialyl Lewis^x is an important tumour-associated carbohydrate antigen present on cell surface glycoconjugates involved in leukocyte migration and cancer metastasis. Radhakrishnan *et al.* reported the formation of sLe^x epitope in butyrate-treated human pancreatic adenocarcinoma cells expressing MUC1 and core 2 *N*-acetylglucosaminyltransferase (C2GnT).²⁸¹ Butyrate treatment stimulates not only the transgene but also a group of endogenous GT genes involved in the synthesis of sLe^x. 5-Aza-2-deoxycytidine increases sLe^x production on MUC1 through induction of ST3Gal VI gene by inhibiting its methylation in colon cancer cell lines.³⁰⁶ Similar results were observed in ovarian cancer, which led to question the validity of using 5-Aza-dC (**63**) as a treatment as potentially facilitates cancer progression and metastasis.³⁰⁷ In a different fashion, cytarabine (**64**) promotes decreased cell surface sialylation in normal human lung tissue (WI-38 cells)³⁰⁸ and acute leukemia (HL-60 cells)³⁰⁹ by inhibiting CMP-Neu5Ac synthetase via the active metabolite araCMP (Fig. 13).

Calnexin and Calreticulin are transmembrane chaperones in the endoplasmic reticulum involved in folding a number of glycoproteins and regulate their uptake in the Golgi apparatus. Calnexin was found to bind to ST8Sia I, the GD3 synthase.³¹⁰ As GD3 is produced by responding to environmental stressors, the inactivation of ST8Sia I contributes to regulate many biological processes such as apoptosis. Significant increase in Calnexin and calreticulin levels were observed in mice with ST3Gal II transgenes.³¹¹

7. BIOLOGICAL TOOLS TOWARDS IMAGING

Labelling and tracking have become techniques of increasing importance in modern biotechnology which are progressively reinforced by advances in material science.³¹² This method enables visualization in cells and organisms as well as the enrichment of specific glycoprotein types for proteomic analysis. In addition to the therapeutic potential of ST inhibitors, labelled sialic acid and sialic acid analogues are also being explored for their diagnostic and prognostic value in imaging sialylation in disease. There are a host of methods for interrogating cell-surface sialylation based either on the affinity of natural sialosides for specific lectins, biosynthetic incorporation of unnatural sialic acids into cell surface glycoproteins that are subsequently detected with complementary labelled substrates, or direct incorporation of a labelled entity such as FITC-Neu5Ac.³¹³

A. Metabolic Glycoengineering

Exploitation of the promiscuous glycosylation biosynthetic machinery has allowed the incorporation of a wide range of unnatural sialic acids and their biogenetic precursors (e.g., ManNAc, Neu5Ac, and CMP-Neu5Ac analogues) into living cells and animals.^{314,315} Once expressed on the cell surface (which can take from just minutes to hours) the functionalised sialic acid (typically bearing a free ketone-, azide-, thiol-, or alkyne-moiety) is subjected to a bioorthogonal chemoselective ligation reaction to a labelled counterpart.³¹⁶ Metabolic glycoengineering is a rapidly expanding field that was pioneered by C. Bertozzi from Stanford and has been reviewed in detail elsewhere.³¹⁵ Herein selected recent examples are included to illustrate the power of this technique in uncovering more about the integral role of sialylation in both disease and normal biological process.

The azide groups being virtually absent in nature constitutes an excellent building block for selectively tagging a biomolecular entity. The ‘clickable’ reagent can be introduced using a cell-permeable biogenetic precursor of CMP-Neu5Ac bearing an azido or alkynyl group.³¹⁷ Once this building block has entered the cell, it utilises the biosynthetic machinery to provide the corresponding sialic acid derivative, allowing the reactive residue to appear as a cell surface modified glycan.^{314,315} This concept efficiently allows the incorporation of a modified moiety on the cell surface. In this manner, a wide range of modified ManNAc analogues have been biosynthetically incorporated into cell surface glycoproteins, with peracetylation significantly enhancing cellular uptake of ManNAc analogues (although it has less of an effect on sialic acid analogues).³¹⁸ In particular, peracetylated *N*-azidoacetylmannosamine, Ac4ManNAz (**65**, Fig. 14), has proven a versatile substrate for copper-free click chemistry³¹⁹ or a Staudinger ligation³²⁰ for dynamic *in vivo* imaging in various animal models including drosophila larvae,³²¹ mice,^{322,323} rat,³²⁴ and zebrafish.^{196,325} For example, Ac4ManNAz has been employed for the site-specific labelling of the heavy chain, *N*-glycosylation site of an anti-CA 19-9 antibody and used for *in vitro* and *in vivo* targeting of pancreatic cancer cells.³²⁶ Ac4ManNAz has also been used to visualise the dynamic sialylation processes occurring during epithelial-mesenchymal transition (EMT) in human keratinocyte HaCaT cells via click labelling with fluorophores and affinity tags. In addition, incorporating the commercially available pan-sialylation inhibitor P-3F_{ax}-Neu5Ac (**50**), it was found that inhibition of global sialylation promoted EMT.³²⁷

Many modified ManNAc and Neu5Ac analogues are commercially available promoting further development of this field. In addition, the corresponding alkynyl sugar derivatives

such as Neu5Hex (**66**) have been applied in an analogous manner to couple with azido-bearing labels (Fig. 14).³²⁸ For example, Homann *et al.* metabolically incorporated Neu5Hex into the cell-surface glycocalyx of a human larynx carcinoma cell line (HEp-2), which was then labelled with a fluorescence tag using click chemistry.³²⁹ Jiang *et al.* utilised peracetylated *N*-(4-pentynoyl) mannosamine (Ac4ManNAI), an alkyne-tagged metabolic precursor of sialic acid, to monitor the kinetics of glycan biosynthesis. Coupling this with an electron-donating picolyl azide and BTTPS, a tris(triazolylmethyl)amine-based Cu(I)-chelating ligand, accelerated efficiency of the CuAAC reaction 20-38-fold. In mammalian cells they reported that it takes 30-45 min for incorporation of ManNAc analogues into cell-surface glycoconjugates, and were able to detect labelled glycans in zebrafish embryos at the two-cell stage (0.75–2 hpf).³³⁰

Photo-cross linking diazirine-modified *N*-acetylmannosamine (ManNAc) have also been incorporated into cell surface glycoconjugates in BJA-B, Daudi, and Jurkat cells.³³¹ Upon exposure to UV irradiation, they were covalently cross-linked to sialic acid binding partners such as Siglec CD22. The compounds were nontoxic and exhibited minimal growth inhibition at the testing concentrations. Expanding on this, dual metabolic labelling of sialylated glycans using bifunctional sialic acid analogues containing two distinct chemical reporters at the *N*-acyl side chain and C9 positions, such as an azide for click labelling and a diazirine for photo cross linking, have been described.³³² The bifunctional sialic acid analogues 9AzSiaNAI and 9AzSiaDAz (**67**) were metabolically incorporated into cellular glycans in Daudi B lymphoblast cells and used for two-colour and FRET imaging (Fig. 14).

B. Fluorescence Imaging

Preidl *et al.*¹⁸¹ developed a series of fluorescent mimetics, *e.g.* **43** of CMP-Neu5Ac based on classical Schmidt type ST transition-state analogue inhibitors **42**.²⁶⁶ The polyanionic fluorescent compounds were efficiently taken up into cells and translocated to compartments surrounding the cell nucleus without the need for transfection agents (Fig. 14). All probes examined were found to bind to the four commercially available STs employed in their study, rST3Gal II, hST6Gal I, and bacterial STs Δ 24PmST1 from *Pasteurella multocida* and Pd2,6ST(N) from *Photobacterium damsela*. All exhibited nanomolar ST inhibitory activity in a fluorescence polarisation based displacement assay with K_i values in the range of 0.66-0.88 μ M against the mammalian STs and 0.08-0.39 μ M against bacterial STs.¹⁸¹

Wu *et al.*³¹³ have recently illustrated the clinical potential for fluorescence guided intraoperative tumour detection using FITC-labelled sialic acid (FITC-Neu5Ac) (**68**), which is

taken up by a broad range of tumour cell lines *in vitro* including Raw 264.7 macrophages, human epithelial carcinoma (HeLa), human hepatocellular carcinoma (QGY-7701), human primary glioblastoma (U87-MG), H22 hepatocellular carcinoma, murine microglial cells (BV-2) and the human embryonic kidney 293 cells (normal tissue). The FITC-Neu5Ac is metabolically incorporated into glycoproteins via a cellular sialylation pathway and transported to the cell surface where it is imaged. Furthermore, intravenous injection of FITC-SA into ICR mice with subcutaneous tumours revealed preferential uptake by tumours *in vivo*, including high target-to-background fluorescence ratios. Live cell imaging using a CMP-Neu5Ac analogue carrying a fluorescent reporter group was also described by Suzuki *et al.* The compound was found to be a substrate of a CMP-sialic acid transporter and enabled visualisation of specific Golgi vesicles.³³³

Non-invasive imaging of intracellular ST activity has been described using tetramethylrhodamine isothiocyanate labeled asialofetuin (TRITC-AF) as a ST substrate and fluorescein isothiocyanate-labeled 3-aminophenylboronic acid (FITC-APBA), which forms a stable chemoselective complex with Neu5Ac at physiological pH. The FITC and TRITC donor and acceptor FRET pair is encapsulated in a liposome vesicle for intracellular delivery. This method can be used to determine ST activity and cell surface sialylation and evaluate correlations with malignancy, as well as assessing activity of ST inhibitors.³³⁴

Figure 14. Examples of sialic acid derivatives or mimics used for imaging.

C. Biomarkers and Biosensors

The over-expression of sialylated epitopes such as sialyl Lewis^a (sLe^a); sialyl Lewis^x (sLe^x); sialyl Tn (sTn); disialylated gangliosides GD2 and GD3; monosialylated gangliosides GM1 and GM2; and polysialic acid (PSA), on the cancer cell surface has been well known for decades and forms the basis of many existing serum tumour markers on the market including carbohydrate antigen CA 19-9 (sLe^a) for pancreatic cancer.⁹ The identification of new highly sensitive biomarkers for early detection and monitoring of disease is a rapidly expanding area and one where a greater understanding of tumour glycosylation and sialylation has immense potential to contribute to the development of new diagnostic methods.^{48,335,336} In addition to these 'natural' tumour-associated carbohydrate antigens (TACAs), the xeno-autoantigen Neu5Gc (**2**), acquired predominantly from red meat diets and detected in high levels in human cancers, also provides a potential target for immunotherapy.^{335,337,338} There are also increasing reports on the development of ultrasensitive biosensors for detecting specific sialylated glycans for cancer diagnosis. These include gold nanoparticle bound *Sambucus nigra* agglutinin (SNA) based sensors that bind specifically to α -2,6-sialylated glycans with detection limits of 0.03 pg.mL⁻¹; Prussian blue-based nanocomposite sensors; and boronic acid-functionalized peptide-based fluorescent sensors with a high specificity for sLe^x cell-surface glycans.³³⁹⁻³⁴¹

In a different glycoproteomic approach, CMP[¹⁴C]Neu5Ac and ST3Gal II were used in an 'exchange sialylation' process to radiolabel and characterise cancer-associated mucin type O-glycans, which resulted in the identification of a unique 25-35 kDa sialylated glycoprotein in pancreatic tumours.³⁴²

8. CONCLUSIONS

Our ever-expanding knowledge of the intricate role of sialylation in cancer, inflammation, immunology and central nervous system disorders, has highlighted the therapeutic potential of this important family of enzymes. Today, an overwhelming number of studies have established the merits of inhibiting specific STs such as ST3Gal I and ST6Gal I, as a powerful new anti-metastatic strategy, with the potential to be effective on drug-resistant tumours. A small number of pan-ST inhibitors are commercially available, however, for ST inhibitors to proceed to the clinical stage, it is essential that selective inhibitors for the different ST subtypes are developed to specifically target those STs over-expressed in cancers and to reduce off-target effects. A wide variety of inhibitors have been developed over the last

two decades both by rational design and from natural product and high-throughput screening efforts. The recent reports of mammalian crystal structures for porcine ST3Gal I, rat and human ST6Gal I and human ST8Sia III, has enabled the use of structure-based design to drive ST inhibitors design in the future to improve inhibitor selectivity and pharmacokinetic profiles. Increased availability of subtype selective, cell-permeable and synthetically accessible, small-molecule ST inhibitors in the future will enable further exploration and therapeutic exploitation of the role of sialylation in a range of diseases, in particular metastatic cancers. In addition to their potential therapeutic role, sialyltransferase inhibitors and their analogues are also being explored as diagnostic tools for disease imaging.

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ABBREVIATIONS

CDP = Cytidine diphosphate

CHO = Chinese Hamster Ovary cells

CMP = Cytidine monophosphate

CMP-3F-Neu5Ac = Cytidine-5'-monophospho-*N*-acetyl-3-fluoroneuraminic acid

CMP-Neu5Ac = Cytidine-5'-monophospho-*N*-acetylneuraminic acid

COX-2 = cyclooxygenase-2

CTP = Cytidine triphosphate

EMT = epithelial–mesenchymal transition

FAK = Focal adhesion kinase-1

FITC = Fluorescein isothiocyanate

FP = fluorescence polarization

FUT3 = Fuc- α -1,3/4-Gal- β -1,4/3-GlcNAc-specific galactoside 3(4)-L-fucosyltransferase

FUT6 = Fuc- α -1,3-Gal- β -1,4-GlcNAc-specific α -(1,3)-fucosyltransferase

FUT7 = Fuc- α -1,3-Gal- β -1,4-GlcNAc-specific α -(1,3)-fucosyltransferase

G6P = glucose-6-phosphate

GT = Glycosyltransferase

GWAS = Genome wide association study

hST = Human sialyltransferase

HTS = high-throughput screening

Lith = Lithocholic acid

LOS = lipooligosaccharide

MDR = Multi-Drug Resistance

MM = multiple myeloma

MS = mass spectrometry

MUC1 = mucin 1

NCAM = Neutral cell adhesion molecule

Neu5Ac = *N*-Acetylneuraminic acid (sialic acid)

Neu5Gc = *N*-Glycolylneuraminic acid

PDB = Protein data bank

pST = Porcine sialyltransferase

rST = Rat sialyltransferase

SAR = Structure-Activity Relationship

ST = Sialyltransferase

Sia6LacNAc = Sia- α -2,6-Gal- β -1,4-GlcNAc

sLe^a = sialyl Lewis^a

SLe^x = sialyl Lewis^x

SSEA4 = stage-specific embryonic antigen 4

ST = sialyltransferase

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