The Therapeutic Potential of Small Pharmacological Molecules in the Treatment of Sialidosis

THE THERAPEUTIC POTENTIAL OF SMALL PHARMACOLOGICAL MOLECULES IN THE TREATMENT OF SIALIDOSIS

BY

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To my family.

Espcially our angel, Mom.

Abstract

Sialidosis is an autosomal recessive disorder caused by a dysfunctional Sialidase enzyme. Categorized into two phenotypes, Sialidosis type I and II, Sialidosis is a highly heterogeneous disorder with varying ages of onset and pathologies. Currently, there is no viable therapy for the treatment of Sialidosis patients. At the molecular level, cells from Sialidosis patients with compound heterozygous mutations show improper enzyme folding, loss of Sialidase enzyme activity and subsequent accumulation of sialylconjugates within lysosomes. One promising treatment option is the use of small pharmacological molecules as immune response, and proteasome regulators. In this study, we examined the efficacy of the immuno-suppressant (Celastrol) as well as a proteasomal inhibitor (MG132) in the rescue of mutant enzymes with altered conformation. Our results reveal that MG132 is highly beneficial to enzyme activity, localization and substrate reduction in cells expressing defective Sialidase. We also found that MG132 reduces accumulation of ganglioside products, GT1b, GD3, and GM3 in pre-loaded Sialidosis cells. Alternatively, Celastrol appears to inhibit; if not deplete Sialidase expression and activity revealing a potentially novel effect of Celastrol on immune modulators. Of interest, the combination of Celastrol and MG132 appears to amplify the beneficial impact of MG132 across most of the molecular analysis of both the endogenous and recombinant expression of defective Sialidase. This study explores a novel biological criteria to assess the efficacy of small molecules through substrate accumulation analysis and points to a potential therapeutic strategy for the treatment of Sialidosis.

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I first wish to thank my supervisor, Dr. Suleiman Igdoura for all of his guidance, mentorship and patience during this project. I am so appreciative of all he has taught me, but most importantly, I recognize the vigor and passion he has for research that he instills in all of his students; including me. He has given me a new future, and for that I will be forever grateful.

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To the crew of 306/321, you are an amazing group of people and there could not be a better group of peers, guides and friends. Thank you all for the support during stressful times, the answers during confusing times, and the constant laughs.

And of course to all my friends who have been completely supportive of my decision to re-enter the scientific world. To Natasha for always pushing me into academics and helping me so much along the way. To my love, Justin, who surprises me every day with his strength and companionship as this stage comes to an end and a new life starts.

Thank you.

List of abbreviations

AP Adaptor Associated Complexes

AdSial Adenoviral Vector expressing Sialidase

ASGP-R Asialoglycoprotein Receptor

Asp box Aspartic box motif

ATF Activating Transcription Factor

BMT Bone Marrow Transplant

CNS Central Cervous System

COOH Carboxy-terminal end of a protein

DMEM Dulbecco's Modified Eagle Medium

EMCV-IRES encephalomyocarditis virus internal ribosomal entry-site

ER Endoplasmic Reticulum

ERAD Endoplasmic Reticulum Associated Degradation

ERT Enzyme Replacement Therapy

G Guanine

GlcCer Glucosylceramide

GRP Glucose Response Protein

GSL Glycosphingolipid

H Histidine

His Histidine

HSC Heat Shock Chaperones

HSCT Hematopoitic Stem Cell therapy

HSE Heat Shock Element

HSF1 Heat Shock Factor 1

HSP Heat Shock Protein

IKK IκB Kinase

LAMP Lysosomal Associated Membrane Protein

LGP Lysosomal Membrance Glycoproteins

LIMP Lysosomal Integral Membrane Protein

LSD Lysosomal Storage Disorder

M6P Mannose 6-phosphate

MAP 2-amino-2-mtheyl-1-propanol

MG132 N-carbobenzoxly-leu-leu-leu-leucinal

MuNANA (4-methylumbelliferyl)-α-D-N-acetylneuraminic acid

MOI Multiplicity of Infection

NBDNJ N-butyldeoxynojirmycin

NEU1 Neuraminidase I: Lysosomal Neuraminidase

NEU2 Neuraminidase 2: Cytosolic Neuraminidase

NEU3 Neuraminidase 3: Membrane Neuraminidase

NEU4 Neuraminidase 4: Mitochondrial Neuraminidase

NFκB Nuclear Factor kappa-light-chain-enhancer of activated B cells

NH3 Amino-terminal end of a protein

P Proline

PERK PKR-like ER-associated Kinase

PBS Phosphate Buffered Serum

pfu Plaque Forming Units

R Argenine

SRT Substrate reduction therapy

TNFα Tumour Necrosis Factor α

UPR Unfolded protein response

XBP X-box DNA binding protein

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Chapter 1

Introduction

1.1 Glycosphingolipids and Gangliosides

Glycosphingolipids (GSLs) are composed of a hydrophobic Ceramide backbone anchored in the lipid bilayer of membranes (Fig. 1.1). The Ceramide backbone has been established as a major component of cellular signalling and apoptosis; as a product of GSL breakdown, this implicates GSLs and in particular gangliosides as a key part of cellular signalling[123]. Gangliosides, a major structural component of neuronal membranes[58] are also associated with somatic cells throughout the body including muscle, platelets and endothelial cells[39]. The sialic acid (N-acetylneuraminic acid) residue found on gangliosides at various different sugar moieties, in particular at the terminal ends prevents Ganglioside degradation into its constitutive parts[42] and is a key component of the signalling ability of gangliosides [113]. Sialic acid can be attached to oligosaccharides, GSLs and glycoproteins through three carbohydrate linkages, the α2-3, α2-6 and the α2-8[118], the latter of which describes the binding of chains of sialic acid, as in Di-Sialo or Tri-Sialo species.

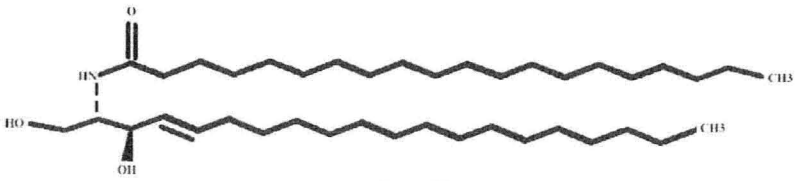
Ganglioside biogenesis is a complex process regulated by a number of glucosyltransferases and sialotransferases that work in the Endoplasmic Reticulum where the sequential addition of sugars creates a variety of gangliosides[44]. The addition of certain sugars is paramount to the specificity of the ganglioside as well as cellular determination and function. Ganglioside biogenesis shown in Figure 1.2 depicts the different series of gangliosides that are generated during metabolism, all originating from ceramide[28,115]. The production of the four distinct ganglioside series: O, a, b, and c, all originate from one common thread, glucosylceramide (GlcCer) which then cascades into a network of species found in both somatic and neuronal cells[45]. The sequential addition of monosaccharides as well as the addition of sialic acid by sialyltransferases occurs in the Golgi apparatus after transport from the ER[35], and determines the nomenclature of each ganglioside species. Nomenclature of gangliosides in the Di-, Tri- and Mono- Sialo series was designated by Svennerholm (1961)

signifying two, three and one sialic acid attached to the monosaccharides of the glycosphingolipids[114]. The sialic acid residue provides a negative charge to the ganglioside making it a polar molecule available for interaction with a variety of signalling molecules and receptors held within the plasma membrane. This polarity makes it possible to isolate and analyse gangliosides by Thin Layer Chromatography, separating each species by charge and size for a better understanding of gangliosides in vivo[80,83].

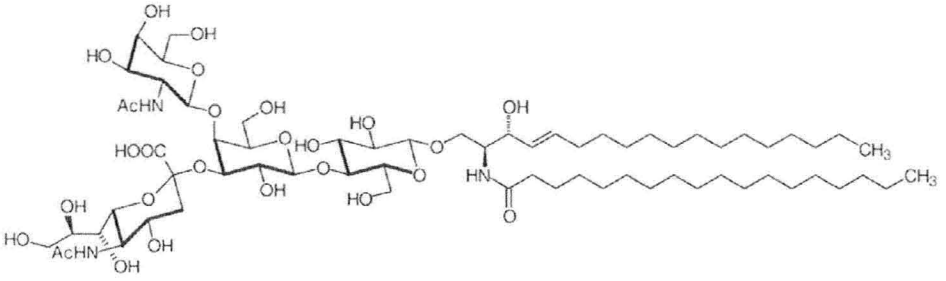
Gangliosides are vital molecules within membranes which interact with a number of cell surface receptors, other GSLs for cell to cell signalling, and transmembrane proteins that regulate cell signal transduction[66,103]. The length and complexity of the saccharide attached to the Ceramide gives the ganglioside flexibility or structure. This structure determines where in the cell the ganglioside will reside, as well as how it will interact with cell surface receptors, pathogens, and ligands; for instance, ganglioside self-interaction within a membrane can change the membrane shape[103].

Gangliosides are found ubiquitously in all cell types; however, the expression of specific gangliosides is increased in certain cell types, for example, GM3 is found abundant in the liver, but is less abundant in the brain as reviewed in Walkley et.al. 2004[120,127]. In cells, gangliosides are catabolised through the lysosomal pathway, where the plasma membrane pinches inward with portions containing glycosphingolipids forming endosomes and multivesicular bodies. The latter matures into lysosomes where their lipid contents are hydrolysed by hydrolases[59]. The subsequent degradation products are recycled back to the Golgi[60]. During GSL catabolism, the hydrolases cleave off terminal sialic acids and sugar moieties and further digest the sugars leaving the ceramide backbone which can be further catabolised to sphingosine and fatty acids[98](Fig. 1.3). Degradation of the gangliosides and sialylated oligosaccharides is a highly regulated event, and in the absence of functional hydrolases we observe accumulations of GSLs and oligosaccharides. The absence of any one of the hydrolases including Hexosaminidase A or B, Sialidase or GM1-β-galactosidase leads to the accumulation of GM1, GM2, GM3 and GA2 in patients resulting in Tay Sachs,

Sialidosis and GM1 gangliosidosis, as reviewed in Kolter and Sandhoff, 2006[60]. Such gangliosides and glycolipids are major biomarkers of disease and a measure of disease severity and progression[127].



Ceramide



Ganglioside GM2

Figure 1.1: **Schematic representations of Ceramide and an example of N-acetyl linked glycosphingolipid GM2.** Ceramide, the backbone structure of gangliosides, as well as a boat representation of a mono-sialo ganglioside, GM2. Image reproduced from Kolter and Sandhoff, 2006[60].

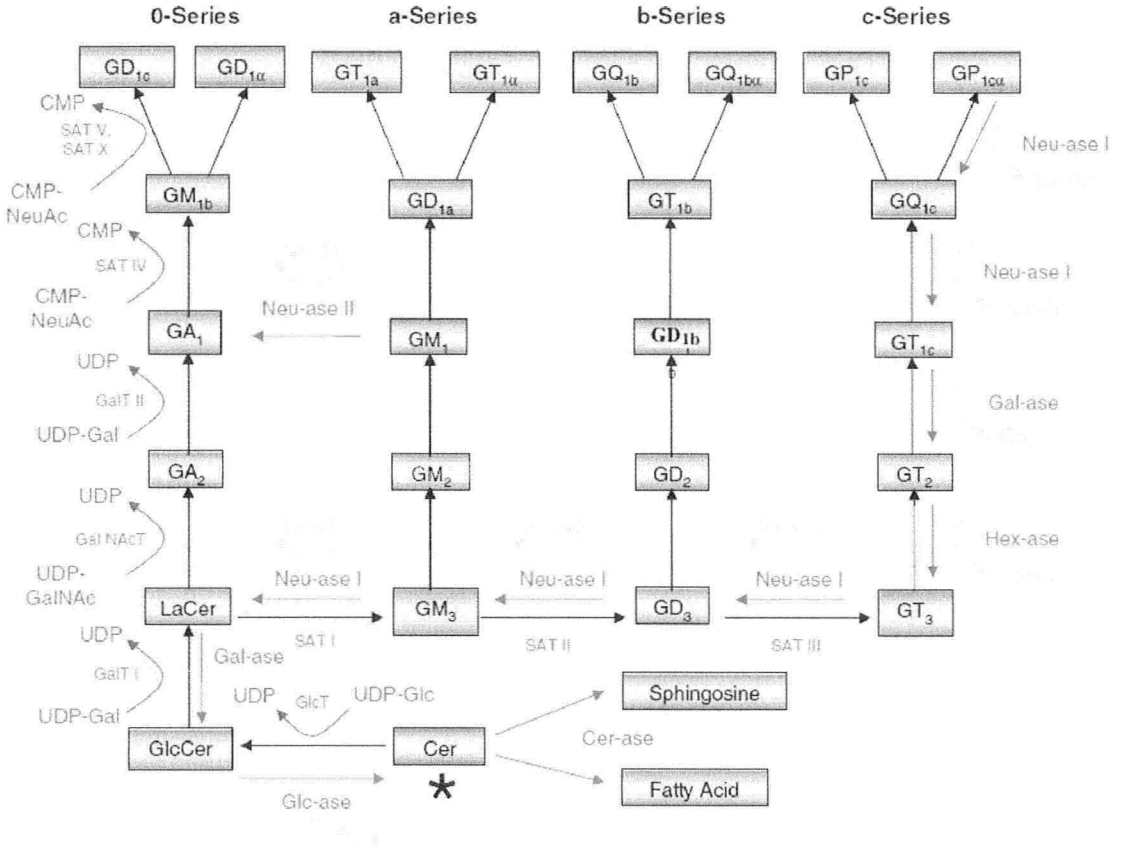


Figure 1.2: **Ganglioside biogenesis.** Through the actions of membrane bound glucosyltransferases within the ER, sugar nucleotides are added to Ceramide to produce simple glycosphingolipids. Transport into the Golgi sees a step wise increase in glycosylation and sialotransferases adding terminal sialic acids to create a complex system of glycosphingolipids to be transported throughout the cell. (Cer) represents Ceramide and is the precursor to all gangliosides. Glycosyltransferases (green) are a part of the metabolism of gangliosides in the Golgi. Glycosylhydrolases (blue) participate in the catabolism of gangliosides in the endosomal/lysosomal pathway. Image taken from D'Azzo et.al., 2006[28].

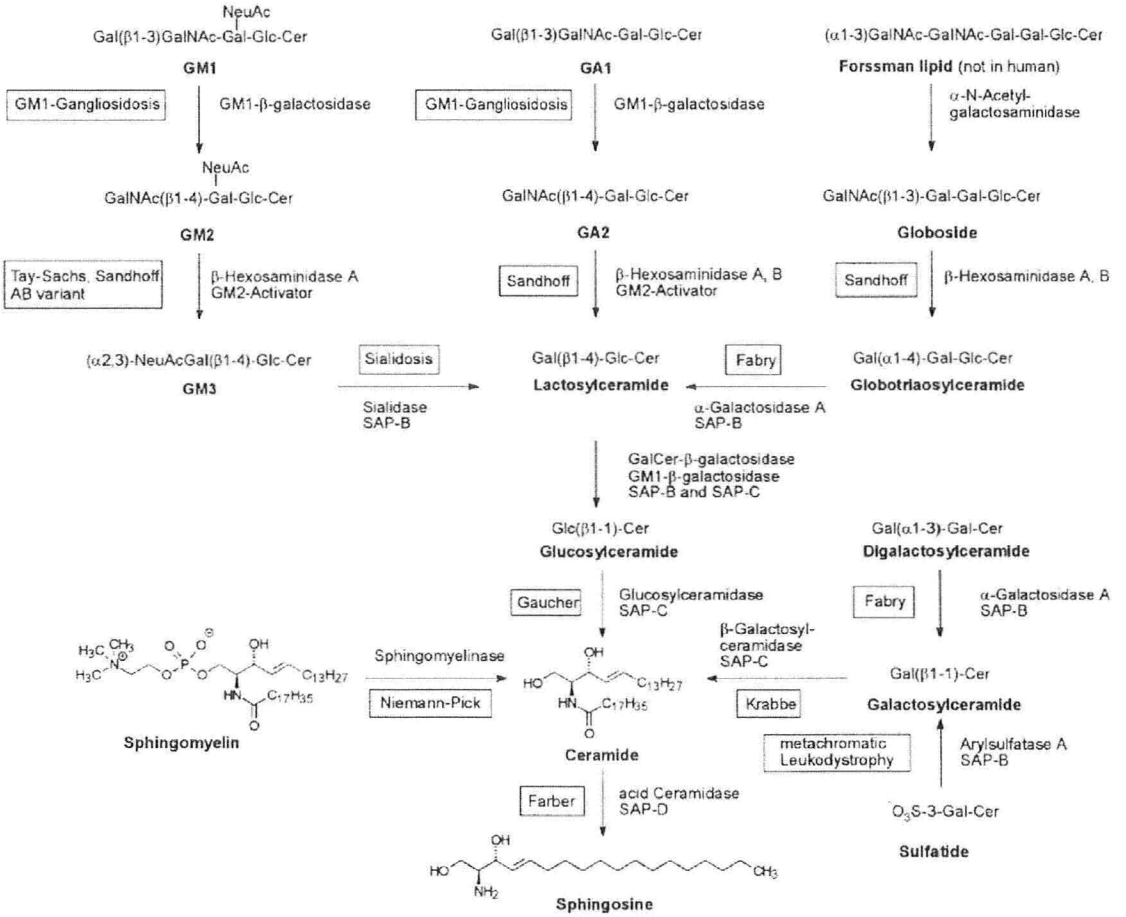


Figure 1.3: **Ganglioside catabolism and associated storage disorders.** Hydrolases within the endosome/lysosome system catalytically cleave off monosaccharides and sialic acid breaking down gangliosides to the backbone Ceramide which is used in the production of sphingosine and sphingomyelin. At any junction if a hydrolase is inactive or absent due to mutation or misfolding there is an accumulation of the substrate characteristic of Lysosomal Storage Disorders. Image taken from Kolter and Sandhoff, 1998[57].

1.2 Lysosomes and Lysosomal Storage Disorders (LSD)

Lysosomes are membrane bound organelles within the cell that contain hydrolases for the degradation of a variety of cellular waste. The membrane of these proliferative organelles contains glycosylated membrane proteins and transport proteins, and is produced by the fusion of late endosomes, plasma membrane and the associated proteins[32]. Also involved in the lysosomal degradation pathway is the cells ability to "eat self" or Autophagy. This is the process wherein the cell delivers cytoplasmic cargo to the lysosome for degradation. In this degradation pathway cellular material like organelles and misfolded protein aggregates are encompassed in a membrane, the matured autophagosome moves to the lysosome and fuses the membranes releasing the cargo to the lysosome for digestion. There is a belief that the autophagosome is ingested by the lysosome and membrane material is degraded and recycled for future use, which is reviewed in Levine et.al., 2008[64].

The lysosomal membrane and luminal environment are unique to the function of this organelle. The membrane is composed of a number of highly glycosylated integral proteins that protect the cell from the hydrolytic enzymes encased within[5]. These proteins, used to identify lysosomes in cells include; the Lysosomal Associated Membrane Protein (LAMPs), the Lysosomal Integral Membrane proteins (LIMP), and to a lesser extent Lysosomal Membrane Glycoproteins (LGPs). These specialized membrane proteins are glycosylated with both N- and O-linked glycans and participate in the stability of the lysosome, membrane and vesicle fusion events, biogenesis of endosome/lysosomes. LAMP II has been associated with chaperone mediated autophagy, while Lamp I has been associated with exocytosis of lysosomes to the plasma membrane[32,136].

The generation of lysosomes is a topic of constant inquiry, as there are a number of theories regarding how lysosomes are made and how they work. Originally, it was believed that lysosomes were generated through "maturation" from early endosomes from the plasma membrane through to the late endosome and mature lysosome. Other schools of thought

support the idea of "vesicle transport" wherein each stage of the lysosomal process; the Late Endosome, Early Lysosome and Mature Lysosome, is its own individual cellular vesicle and transportation occurs between them. Finally, there is the Kiss-and-Run theory of lysosomal degradation wherein there is a fusion and fission of late endosomes with lysosomes where they exchange their contents but maintain stability (Fig. 1.4)[82]. The Kiss-and-run theory is fortified in experiments using gold labelling to track lysosomal material transport, and it was determined that the Late Endosome and Early Lysosome both maintained gold beads after a chase, and hydrolases were also found throughout the endosomal/lysosomal pathway [116]. Understanding the complexity of the endosomal/lysosomal process allows us to fully comprehend the degradation products and recycling pathway of the cell.

The lysosome has been targeted as the main source of protein and glycosphingolipid degradation within the cell. Understanding how lysosomes degrade these products reveals potential therapy targets during times of dysfunction. Hydrolases are generated in the Golgi Network where they are processed and targeted to the lysosome through clathrin coated pits, or the Mannose 6-Phosphate receptor pathway[68,71,95]. Research points to a gradient decrease in pH from 6.7 eventually to below 5 as the hydrolases are transported through the late endosome and early lysosome to the mature lysosome. Once in the early endosome enzymes and ligands are disassociated from their membrane bound receptors (i.e., Mannose 6-Phosphate receptor), which is recycled back to the plasma membrane or the trans-golgi [68]. Hydrolases within the late endosome are exposed to the decreasing pH levels and the pro-enzyme that was delivered in the early endosome begins to mature to the active enzyme. This maturation of enzymatic activity until the mature Lysosome gives a gradient of activity starting with degradation of the less complex ligands finishing with the stable ligands[116].

The function of the hydrolases found within the lysosome is highly regulated and any disruptions in these processes lead to the group of disorders known as the Lysosomal Storage Disorders (LSD), hallmarked by the accumulation of undigested substrates in large vacuoles within the cell. Amongst these lysosomal storage disorders is Sialidosis, resulting

in an accumulation of sialylated oligosaccharides and gangliosides after malfunction in the lysosomal hydrolase Sialidase (Neuraminidase I).

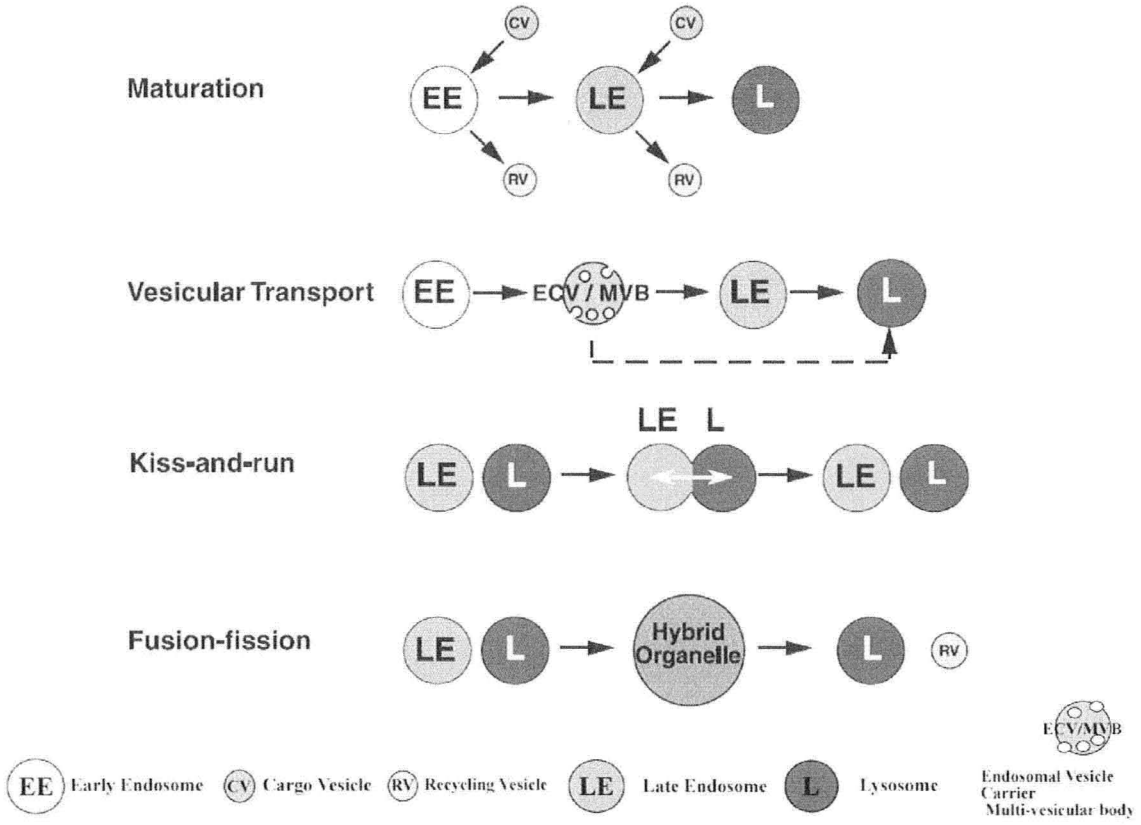


Figure 1.4: **Schematic outline of the four theories of Lysosomal biogenesis.** Theories about the development of the acidic lysosomal environment follow four general methods. Hydrolases made in the ER/Golgi system, as well as materials from the Plasma Membrane or other organelles in the cell are all transported to the lysosome to be degraded and recycled for future use. The generation of the late endosome and how materials are delivered to the highly acidic environment continues to be debated. Image taken from Mullins and Bonifacino, 2001[82].

1.3 Sialidosis (Mucolipidosis I)

Sialidosis is a rare autosomal recessive lysosomal storage disorder (OMIM #26550) resulting from malfunctioning Lysosomal Sialidase which is encoded by the Neu1 gene[8,17,46]. Mutations in the Neu1 gene lead to a defective enzyme which cleaves isalic acids from oligosaccharides, glycolipids and GSLs[96]. Sialidosis is categorized into two distinct phenotypes; Sialidosis Type I and Sialidosis Type II[67]. Type I, late onset (between 8-10 yrs of age), is identified by a macular Cherry-Red Spot characteristic of the lysosomal storage diseases, as well as a decrease in visual acuity and declining hearing loss[134]. Patients in this group generally live into their 20s and are diagnosed with an increase in sialyloligosaccharides as well as vacuoles in blood smears[29,91]. Type II is further subdivided into the infantile and juvenile subsets, determined by the age of onset, maturation of symptoms and symptom severity. Although type II generally is a more severe disorder than type I there is a great deal of variation within this subset[67].

Infantile Sialidosis is generally distinguished very early in life if not in utero. In general patients in this group have a severe onset of symptoms and are deceased by the 5th year of life[55]. Juvenile Sialidosis is marked by a later onset of disease and generally have a longer life span and symptoms that lessen in severity[91]. Type II Sialidosis has hallmark clinical features including course Hurloid features, ataxia with myoclonus jerks, hepatomegally and splenomegally[55,109,111]. This severe phenotype is accompanied by varied levels of mental retardation and death at an early age and is reviewed in Lowden and O'Brien, 1979[67]. The most devastating onsets of disease are neo-natal resulting in hydrops-fetalis[6,119]. Clinical diagnosis begins with the observation of the Cherry Red Spot myoclonus, and the recognition of an abundance of α2-6 linked sialyloligosaccharides[17,29].

The discovery of the accumulation of sialylated glycoconjugates by Cantz et.al., 1977 pointed to Sialidase, the catalytic enzyme that cleaves sialic acid from polysaccharides, as the cause of this disease[17]. Later it was determined that a number of Neuraminidases

existed in the cell and that each had their own substrates and functional locations in the cell[1,67].

The Sialidases are the group of hydrolases that work on sialic acids attached to glycosphingolipids, glycolipids and oligosaccharides. Four human Sialidases have been characterized to date; the lysosomal (NEU1)[8,89], cytosolic (NEU2)[78], membrane bound ganglioside specific (NEU3)[77], and the mitochondrial (NEU4)[76,135]. Each of the Sialidases is unique in its substrates and subcellular localization; however, they have highly homologous amino acid sequences including a F(Y)RIP motif at the amino terminal and a series of ASP boxes (Ser/Thr-X-Asp-(Xb-Gly-X-Thr-Trp/Phe) throughout the protein sequence[8,69]. These homologous sites are potentially involved in the active site recognition of sialic acid[69]. Neu1 gene encodes the lysosomal Sialidase identified to contain mutations in Sialidosis patients and SM/J mice[48]. The characterization of Sialidase shows tissue localization with varying expression levels in the kidneys, epididymis, brain and spinal cord, adrenal, liver, lung, spleen and heart with the most expression in the kidney and least in the heart, liver, lung and spleen[8,48]. Originally described as the lysosomal Sialidase because of the trademark association with the lysosomal marker LAMP II[8,48] we now know that Sialidase can also be targeted to the plasma membrane and participate in regulation of sialylation at the cell surface for signalling, cell to cell recognition and immune signalling[3,43,71,112]. The nature of this protein and its variety of purposes denotes the significance of the lysosomal Sialidase, which is the only Sialidase currently associated with disease.

Sialidase is part of a multi-protein complex consisting of Protective Protein Cathepsin A and β-galactosidase. The complex is formed in the ER and Golgi and is required for Sialidase activity. It also maintains stabilisation and prevents dimerization of Sialidase [10,11,69,70]. The complex is well characterised and the putative binding sites for complex formation have been postulated[10,70].

Mutations in Neu1 have been characterized with over 40 known mutations leading to Sialidosis in varying degrees of severity and are reviewed in Seyrantepe, 2003[105]. Mutations

in Neu1 include splice-site mutations, nonsense mutations leading to premature stop codons, and missense mutations leading to misfolded proteins and are reviewed in Seyrantepe, 2003 [105]. All mutations lead to unique variations of phenotype ranging from hydrops-fetalis to mild clinical symptoms. Understanding these protein changes gives a better understanding of the downstream effects of each mutation on the proper processing, folding, and targeting of Sialidase. Analysis of a number of different missense mutations have been extrapolated to the putative tertiary structure of Sialidase using homology sequences of NEU2 as well as bacterial Sialidase[49,70,73,86]. This putative tertiary structure is used to establish the potential effects mutations have on Sialidase activity and identify targets for therapeutic studies. Through homology analysis, we can determine putative binding sites for ligands as well as the potential complex formation sites that are affected by mutations in patient samples.

1.4 Endoplasmic Reticulum Associated Degradation and the Unfolded Protein Response

The endoplasmic reticulum (ER) is the organelle wherein glycoproteins are synthesized and post-translationally modified and sent to the Golgi for further processing[54]. As a key organelle in proper cellular function, the ER responds to stress in a very controlled and efficient manner. Stress in the ER can come about because of a number of problems including but not limited to: misfolded proteins, inhibition of N-linked glycosylation, reduced calcium stores in the ER lumen and the over-expression of any glycoprotein[54]. In response to these stressful stimuli the cell initiates a cascade of events known as the Unfolded Protein Response (UPR). This aptly named process is a series of translation and transcriptional regulations allowing the cell to handle the influx of proteins into the ER.

The UPR cascade involves both transactivators and ER-resident chaperones. The characterization of this process was elucidated using homology of mammalian cells with the well characterized Yeast response to stress. In mammalian cells, stress levels initiate the transducers IRE1, PERK (PKR-like ER-associated kinase) and the ATF6 (activating transcription factor-6); all of which initiate the UPR when the luminal extensions sense stress in the ER[138]. Transducer activation is initiated when the glucose-regulated protein, Grp78 (BiP) is released from the ER luminal end of the transducer to act as a chaperone for any misfolded proteins in the ER[7]. The release of BiP allows for activation of PERK, IREI or ATF6, each of which is activated in different cell types and under different cellular conditions, although the method by which this regulation occurs is still under investigation[7]. PERK phosphoprylates eIF2 leading to an overall reduction in translation to help reduce the ER workload and allow the ER machinery to clear any backlog[101]. ATF6 is known to bind to ER stress Response Elements (ERSE) that are found in both yeast and mammals, with a consensus sequence of CCAATNgCCAGG located in regions of GRPs and ER regulation machinery. Binding of this element by ATF6 is increased in stressful situations and

increases the UPR machinery[137]. Eventually, chronic UPR signalling leads to cell death[101].

Following the initiation of the UPR is the ER stress-associated degradation pathway (ERAD), a process by which misfolded proteins are tagged, and translocated to the cytoplasm. In the cytoplasm misfolded proteins are ubiquitinated by ubiquitin-ligases and targeted to the 26s proteasome for degradation[131]. The initiation of ERAD begins when BiP is released from the IRE1 transactivator; upon release from BiP, IRE1 phosphorylates itself activating its transcriptional regulatory activity. IRE1 can then post-translationally process the X-box DNA binding protein (XBP1), reformatting it to efficiently translate proteins involved in ERAD including BiP and GRP94 required for the activation of ERAD [106].

ERAD and UPR signalling has become an interesting target for therapeutic treatment of disorders of misfolded proteins like Sialidosis. There is keen interest in understanding the process involved in the accumulation of proteins through this system and exploiting the machinery involved in the regulation of protein folding and transport[74].

1.5 Treatment Opportunities for Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are marred with a number of characteristics that hinder treatment of LSD patients. There are over 40 known lysosomal storage disorders, and amongst them there are a variety of cell types, organs and substrates that are affected [36]. The variability of LSDs is not limited to the overall group but within each disorder there are a number of identified mutations and malfunctions that increase variability of phenotype; Sialidosis has over 60 known mutations [105]. The high variability creates a number of problems when designing treatment options that can work across the entire spectrum of phenotypic characteristics of Lysosomal storage disorders.

There have been successful therapies for some lysosomal storage disorders focused on symptom management but none that have successfully reversed symptoms already onset [5]. For example, the use of Enzyme Replacement Therapy (ERT) in lysosomal storage disorders has been used with some success. This is the process by which a recombinant enzyme is administered to patients and the cells take up the enzyme from the extracellular space. Most of the recombinant enzymes take advantage of the Mannose 6-phosphate (M6P) receptor transport pathway used by the lysosome to receive soluble enzymes [5,34,68]. Success in ERT has been seen with Gaucher, Pompe and Fabry disease [14,19,33]. Aside from the enormous cost of this treatment, ERT has a series of complications and drawbacks as a therapy for some LSDs. First, the up-take of exogenous enzymes through the M6P is not highly efficient for all cell types; in fact, hepatocytes alternatively utilize the asialoglycoprotein receptor (ASGP-R) recognizing terminal galactose for uptake of exogenous material [133]. Also the number of M6P receptors is so low in brain and skeletal muscle [130] requiring high concentrations of exogenous enzyme in order to promote substrate clearance. Alternatively, the underlying problem in LSD could be the proper trafficking of hydrolases using the M6P receptors, as is seen in I-Cell disease,therefore, enzyme delivery through this receptor is impossible [5]. There

is also the potential that patients will develop neutralizing antibodies to the foreign enzyme reducing efficacy and mounting a potential immune effect[41]. Finally in neurodegenerative cases, like Sialidosis, the blood brain barrier poses an enormous problem for delivery of enzymes to the brain for clearance of substrates in neurons[41].

Similar to ERT, Substrate Reduction Therapy (SRT) is also employed to treat LSDs. Substrate reduction therapy employs certain inhibitors to prevent the production of accumulating substrates by blocking the biogenesis pathway. There has been success using N-butyldeoxynojirmycin (NBDNJ), a potent inhibitor of α-glucosidase I and II, or Miglustat in Gaucher patients[26,31,50,87]. These treatments are promising because of the small nature of the pharmacological inhibitors being used, as they may cross the blood brain barrier to gain access to the brain and prevent further damage to the CNS[5].

Correction of genetic mutations has been explored with mixed levels of success. The use of Hematopoietic Stem Cell Therapy (HSCT) or Bone Marrow Transplant (BMT) is a useful treatment option early in diagnosis, however the proceedure itself involves risk and is not always effective[5]. Gene therapy is currently under review and there has been success in animal models[22,23]. LSDs are good models for gene therapy because they are typically monogenic. Gene therapy to date does surpass ERT or SRT in the ability to be a sustained therapy treatment[22,52]. However, gene therapy also does not ensure that treatment can cross the blood brain barrier unless injected into the brains of animals producing severe immune response side effects, making it mandatory to accompany treatment with immune suppressors[23] which carry their own side-effects. Also a retroviral delivery system can result in transgenes inserted into a functioning gene, or cause mutagenesis[5].

Currently under investigation is the use of enzyme enhancement using small pharmacological chaperones or proteosomal inhibitors to increase the quality of enzyme being produced by the diseased cell. Although the use of chaperones does not have the impact that gene therapy and ERT has, an increase of a mere 5% enzyme activity can improve phenotype. In fact, a number of previously identified inhibitors have now been identified as

possible chaperones because of their binding efficiency to their target substrates, and their ability to work on a number of substrates and be released in the acidic environment of the Lysosome[93,117]. A number of LSD mutations are missense mutations leading to misfolding [105], and these alleles are highly susceptible to the use of pharmacological chaperones. As a result, research is branching into the investigation of proteosomal regulators and pharmacological agents that may reduce the ERAD and UPR response. Some success has been seen in Gaucher and Tay Sachs disease mutations[81] wherein these agents improved endogenous enzymatic activity and improved cellular function[15]. This latest treatment option has many potential possibilities as treatment could be less mutation specific and allow for global treatment of these highly variable disorders.

1.6 MG132 and Celastrol as Potential Therapeutics

With treatment options being so limited in Sialidosis patients, there is keen interest in identifying novel mechanisms to improve enzymatic action on accumulating substrates. The use of proteosomal regulators in Gaucher and Tay Sachs patient cell lines was promising since it results in significant increases in enzymatic activity and lysosomal localization across multiple mutant enzymes for both diseases[81]. For this reason, we investigated the potential of using proteosomal regulators on Sialidosis mutant enzymes in hopes of establishing a potential treatment for Sialidosis patients.

MG132 is a reversible, highly specific, and potent proteosomal inhibitor, arresting the chemotrypsin-like activity of the 26s proteasome, a rate-limiting site in protein degradation. MG132 is primarily used to investigate the potential role of proteosomal activity in the cell; however, this regulator is now investigated for its treatment potential in cancers[62]. MG132 (N-carbobenzoxly-leu-leu-leu-leucinal) (Fig. 1.5) prevents the catalytic activity of the proteasome through reducing activity of the βsubunits (particularly $\beta 5$ and $\beta 1$) of the 20s core of the 26s proteasome[27]. Naturally, reducing the proteosomal pathway has a number of downstream effects including an increase of machinery involved in ERAD and the UPR including BiP and HSC 70[15]. With the increase in folding machinery there is the potential of MG132, or other similar proteosomal inhibitors to allow for proteins normally degraded by the proteasome to be processed through the ER and to be transported to the Golgi and then Lysosomes.

Celastrol is a compound derived from the "Thunder of God Vine" (*Tripterygium wilfordii*) and has been used for a number of decades as an anti-inflammatory drug in patients suffering from Rheumatoid Arthritis and Asthma, as reviewed in Salminen et.al., 2010[97]. Celastrol is a tripterine (part of the family of terpinoids) (Fig. 1.5) and is extensively studied for its effects on the immune mediated signalling pathways as well as effects on

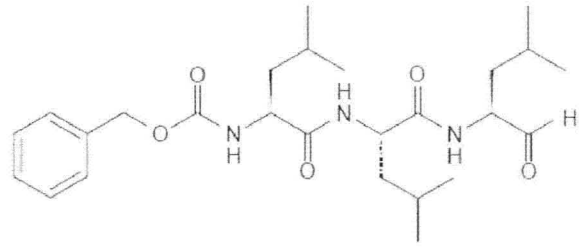
angiogenesis and cell death in cancers. Primarily, Celastrol is able to nucleophillically attack the thiol (R-S-H) group of cysteine interrupting connections with active site residues and disulfide bonds[97]. Celastrol's effect on the immune signalling pathway is attributed to the reduction seen in NFκB which initiates transcription of a number of genes involved in both the adaptive and innate immune responses. Celastrol prevents phosphorylation of the IκB kinase (IKK) through interaction with the Cysteine 179[51,63,84,97]. Celastrol is also connected with repression of Tumour Necrosis Factor (TNFα), a known NFκB activator, giving an alternative pathway to NFκB inhibition and subsequent control of the mediators of the immune response[2,24].

As a quinine methide triterpenoid, Celastrol produces significant increases in the chaperones and machinery linked to ERAD and the UPR, primarily affecting the cytoplasmic Heat Shock Proteins (Heat Shock Chaperones) like HSP 70 and HSP 90 Celastrol activates Heat Shock Factor 1 (HSF1)[132], or interacts with co-chaperones like cdc37 to initiate ERAD responses[21,84]. Therefore, Celastrol is a promising target for treatment of disorders of misfolded proteins.

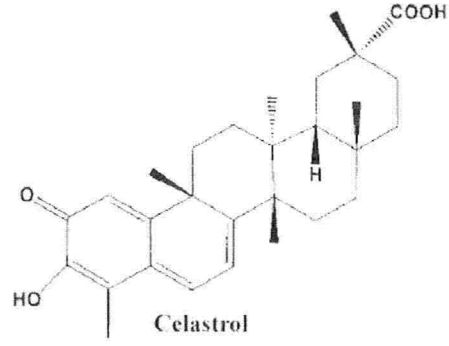
Inflammation and protein accumulation is linked in neurodegenerative diseases like Alzheimer's, Parkinson's, and ALS[4]. Celastrol's effects on the inflammatory mediators, such as TNFα and NFκB have been exploited in the reduction of amyloid plaques and aggregating proteins in Alzheimer's, Parkinson's, and Multiple Sclerosis[2,24,84,97]. In addition, there has been evidence that Celastrol can interact directly with Huntingdon protein to prevent aggregations and reverse phenotype associated with disease state[128]. This discovery provides evidence that this tripterine acts as a chaperone as well as initiates the ERAD machinery needed to prevent misfolded proteins from aggregating in the cytosol.

Taken all together Celastrol proves to be a highly informative and potentially beneficial treatment opportunity for patients suffering from neurodegenerative disorders and protein aggregation disorders. The combination of Celastrol as a potent anti-inflammatory drug and MG132 as proteosomal regulator could provide a novel approach to the increasing substrate

accumulation in Sialidosis patients.



MG132



Celastrol

Figure 1.5: **Schematic diagrams of proteosomal inhibitor MG132 and anti-inflammatory regulator Celastrol.** Mg132 is a common proteosomal regulator that is highly specific for the 20s proteasome. Celastrol is highly susceptible to nucleophillic attack from the thiols of cysteine and is a recognized inhibitor of inflammatory modulators TNFα and NFκB. Image taken from Jin et.al., 2002 and Lee and Goldberg, 1998[51,62].

1.7 Research Objectives

Sialidosis is a rare autosomal lysosomal storage disorder with severe consequences for patients including hurler phenotype, hepatomegally, splenomegally and even myoclonic epilepsies[107,108,110]. Treatment options for such patients are limited to symptom control; identification of potential targets for treatment studies can be highly beneficial. In ascertaining how treatment plans may affect the phenotypic symptoms of specific mutations of Sialidase we aim to identify potentially global treatment options. The clarification of the phenotype-genotype relationship is an ongoing investigation for research surrounding Sialidosis. Characterizing accumulating substrates and clarifying the downstream effect of specific mutant alleles brings us closer to future treatments.

1.7.1 Objective One:

To establish the impact of pharmacological agents on the functional rescue of mutant Sialidase in Sialidosis cell lines, we will examine the effect of drug treatment on enzyme activity, lysosomal targeting and substrate accumulation in cells from Sialidosis patient, and in adenoviral mediated expression of Sialidase mutant alleles in Sialidase-null fibroblasts.

1.7.2 Objective Two:

Define the accumulation of gangliosides, particularly GD1a, GD3 and GM3 in Sialidosis patient fibroblasts and cells expressing individual mutant alleles of Sialidase. We will also explore any reduction of accumulation after treatment with pharmacological agents.

Although this project is the beginning of an investigation of proteosomal inhibition and anti-inflammatory regulation as potential therapy targets for Sialidosis patients; ultimately, we hope to identify novel therapeutics to target the spectrum of symptoms and severities that accompany this devastating disorder.

Chapter 2

Materials and Methods

2.1 Cell Lines

For studies involving allele specific mutations of Sialidase human Sialidase-null fibroblasts (WG544) were obtained from the Montreal Mutant Human Cell repository. Primary patient fibroblasts (H1) were used to examine endogenous Sialidase of a Sialidosis patient. H1 cells were characterized previously as heterozygous for the c.3G>A (p.M1?) and c.1021C>G (p.R341G) mutations of Sialidase[86]. For examination of Sialidase activity in normal human fibroblasts, MCH64 cells were obtained from Montreal Mutant Human Cell Repository.

2.2 Chemicals and Antibodies

MG132 and Celastrol were obtained from Calbiochem (Calbiochem, #474790, #219465), dissolved into 70% ethanol at a 1mM stock concentration and stored at -20°. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, #11965-092) was used as culture medium for all cell types and was supplemented with 10%FBS, 1%PenStrep and 0.1% Fungizone (Invitrogen). Cells were passaged using 1× Phosphate Buffered Saline (PBS) (Gibco) and 0.25% Trypsin-EDTA (Invitrogen). For ganglioside loading of fibroblasts, cells were grown in OptiMEM (Gibco, 31985-070) supplemented with antibiotics and Fungizone. For adenovirus infection for allele specific studies of Sialidase, adenovirus preperations previously generated (Pattison et al, 2005) were suspended in PBS++; made using 500ml of sterile 1× PBS combined with 5ml each of autoclaved 1%$MgCl_2$ and 1%$CaCl_2$.

Mouse monoclonal antibodies against human LAMP II were obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD). Polyclonal rabbit anti-human sialidase antibody described previously was used to identify the carboxyl (COOH) terminus of the Sialidase protein. Mouse anti-RGS His (Qaigen) recognizes a poly-histidine tag added to the N-terminus (NH2) of recombinant Sialidase[47] and was used in experiments examining allele specific Sialidase. Polyclonal goat anti-human Grp78 (N-20) used in western blot

analysis was obtained from Santa Cruz (#sc-1050). Polyclonal goat anti-mouse β-Actin antibody used for normalizing protein levels was acquired from Cell Signalling (Cell signalling, #3700). Alexa Fluor594 goat anti-mouse IgG and Alexa Fluor448 goat anti-rabbit IgG were purchased from Invitrogen (#A11005, #A11034) for allele specific immunolocalisation studies. The goat anti-rabbit bodipy-green-Fl and goat-anti mouse Texas Red-conjugated IgG purchased from Molecular Probes (Eugene, OR), were used in establishing immunolocalisation of endogenous intracellular Sialidase. Nuclei were stained using Hoechst nuclear stain. Goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP were purchased from Santa Cruz, CA (#sc-2005, #sc-2004, #sc-2020).

The specific activity of Sialidase was determined using the artificial substrate -(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MuNANA) (Sigma, #058K5002) and 0.1M 2-amino-2-mtheyl-1-propanol (MAP). For characterization of ganglioside catabolism of both endogenous Sialidase and allele specific Sialidase, fibroblasts were incubated with a bovine brain mixture of gangliosides purchased from Calbiochem (Calbiochem, #345717) and suspended in 70% ethanol at a 2mg/mL stock concentration.

2.3 Cell Culture

Fibroblasts were grown in DMEM at 37° and 5% CO_2 in 75cm^2 vented cap culture flasks (Sarstead) until 90% confluent. Cells were passaged every 2 to 3 days by washing cells in PBS, and incubating for 5 minutes in warm 0.25% Trypsin-EDTA. Cells used in experiments were split evenly (by volume) across 6 or 24 well plates and allowed to adhere for 24 hours before further treatment. Cell counts for adenovirus infection were performed using a hemocytometer and inverted Zeiss microscope.

2.4 Adenovirus Infection

For examination of Sialidase cDNA expressing specific missense mutations, an adenovirus mediated expression of Sialidase was employed. Adenoviruses previously generated[86] at a high titer (1×10^9pfu/ml) express sialidase cDNA containing missense mutations leading to protein substitutions at R341G (c.1021C>G) or R225P (c.674G>C). Viruses expressing mutant and wild type Sialidase were suspended in sterile PBS++ at a 1×10^8pfu/ml for infection. Cells were infected with 10, 100 or 200 MOI of the viruses expressing the R341G mutant ($AdSial^{R341G}$), R225P mutant ($AdSial^{R225P}$) or normal ($AdSial^{+}$) Sialidase. $AdSial^{CIRES}$ was co-infected with $AdSial^{+}$ in ganglioside studies at an MOI of 10. After 30 minutes incubation at 37° and 5% CO_2, DMEM was added to the cells and they were returned to 37° for 24 hours before further treatment.

2.5 Immunolocalisation Studies

To determine the intracellular localization of Sialidase in cells after treatment with MG132, Celastrol or a combination of both MG132 and Celastrol, cells were grown on round glass coverslips (VWR) in a 24 well plate (Sarstead). For investigation of allele specific mutant analysis, cells were infected with $AdSial^{+}$, $AdSial^{R225P}$ or $AdSial^{R341G}$ followed by drug treatment after 24 hours. For examination of endogenous Sialidase localisation, Sialidosis cells were treated after plating. MG132 and Celastrol were suspended in DMEM at a concentration of 0.6μM either independently or in combination. After treatment, cells were incubated for 72 hours at 37° and 5% CO_2. Coverslips were washed with 1ml of 1× PBS in the 24 well plate. Cells were fixed with 150μl of 3.8% formaldehyde in PBS for 30 min at room temperature. After washing in 1ml of 1× PBS, cover slips were permeabolized using 500μl of 0.5% Triton-X100 in PBS for 30 min at room temperature. Slides were washed with 1ml of PBS and blocked in 150μl of 20% Goat Serum in PBS for one hour at room temperature on. Following blocking, cells were incubated with: 100μl of 1× PBS as a secondary only

control, 100µl of goat anti-rabbit Sialidase antibody, or 100µl of goat anti-mouse LAMP II antibody or 100µl of both the polyclonal rabbit anti-sialidase and monoclonal mouse anti-LAMP II at a concentration of 1:400 (Ab:PBS) overnight at 4°. The next day, cells were washed with 500µl of 0.05% TWEEN in PBS four times for five minutes at room temperature. Secondary fluorescent antibodies were suspended in PBS at a concentration of 1:400 and centrifuged at 16,400 rpm for 10 minutes to remove any unbound fluorophore. Cells incubated with a single primary antibody were stained with 125µl of the corresponding secondary to control for fluorescent cross-reactivity. Slides used in allele specific studies were stained with the anti-mouse Alexa Fluor594 to recognize LAMP II and anti-rabbit Alexa Fluor488 to visualize Sialidase. Cells used in endogenous studies of Sialidase were stained with the anti-mouse Bodipy Green-FL for identification of LAMP II and the anti-rabbit Texas Red fluorescent secondary for identification of Sialidase. While protected from light, slides were incubated with secondary antibodies at room temperature for one hour. After secondary antibody incubation, cells were washed with 500µl of 0.05% TWEEN in PBS four times for five minutes at room temperature to wash off unbound antibody. To identify nuclear material, slides were incubated for 10 minutes at room temperature with 500µl of Hoechst nuclear stain in PBS (1:1000) (AB:PBS). Slides were washed with 1ml of PBS followed by dipping the slide in double distilled water. Slides were mounted cell-side down on microscope slides (VWR) using proLong©gold antifade reagent (Invitrogen, #P36930). Mounting medium was allowed to harden for 24 hours in the dark before cells were imaged. Slides were stored at -20° in a slide box to ensure longevity of fluorescent staining.

For immunolocalisation studies, slides were analyzed on the LeicaTCS SP5 inverted confocal microscope using the 63× 1.3NA DIC glycerol HCX Pl-Apo objective. Each field was imaged using the lasers: 488 nm Argon ion, 10 mW 561 nm DPSS, and Chameleon Ultra 690-1040 nm 2.5W at 800 nm to capture green, red and blue fluorescents respectively. Laser intensity and wavelength controls were determined on an experimental basis using the secondary only, and individually stained slides as controls. Coloured overlays were generated

using the Leica LAS LF software. Each treatment was performed on two slides and images are representative of the entire population of cells in each treatment group. Complete image sequences were pseudocoloured using ImageJ software (McMaster Biophotonics) and adjusted for print using Photoshop.

2.6 Quantification of Immunolocalisation Studies

Original confocal images were opened in ImageJ (McMaster Biophotonics) software for analysis as .TIFF images. Any background fluorescence within individual Z-stacks was adjusted using the *region of interest (ROI) background subtraction* plug-in in ImageJ (McMaster Biophotonics). In a single z-stack, cells in the green channel image were outlined using the freehand tool and added to an ROI manager in ImageJ. Whole cells within a field of view devoid of cross-over from other cells were used for quantification analysis. The green pixels and red pixels for each z-stack were quantified and applied to Mander's Coefficient:

$$R = \frac{\sum_i (R_i \times G_i)}{\sqrt{\sum_i (R_i)^2 \times \sum_i (G_i)^2}}$$

using the *Mander's ROI manager* plug-in in ImageJ (McMaster Biophotonics). The Mander's Coefficients for each z-stack were summed for every cell and represent one value in quantification. Five to 14 cells were analyzed from at least two fields of view on each slide. Statistical analysis of each treatment was performed using a one-way anova followed by the Dunn's multiple comparison test. Endogenous levels of Sialidase colocalisation was quantified using the Mander's Coefficient from a single optical slice of a minimum of three cells from at least two fields of view.

2.7 Ganglioside Catabolism Studies

To characterize ganglioside catabolism *in vitro*, fibroblasts were incubated for five days with and without 200µg per flask of bovine brain gangliosides. Treatment with 0.6µM MG132, 0.6µM Celastrol or a combination of 0.6µM MG132 and 0.6µM Celastrol in DMEM followed ganglioside loading. In allele specific analysis, adenoviral infection with AdSial^{+}, AdSial^{R225P} or AdSial^{R341G} preceded drug treatment. After 72 hours of treatment, cellular material from four 75cm^2 flasks was pooled into one sample and harvested in water. Lysates in water were sonicated at 20% pulse, transferred to glass conical tubes and suspended in chloroform/methanol/H_20 (C:M:H_20) (10:10:1 v:v). Further sonication in a 4° water sonicator using a 15 minute program (20s on, 20s off) was followed by centrifuging for 15 min, 3000rpm at 4°. The supernatant was collected leaving a pellet which was re-suspended in C:M:H_2O (10:10:1), sonicated using both the pulse sonicator and water sonicator, and centrifuged again collecting the second supernatant. The final pellet is suspended in C:M:NaAcetate (0.8M) (30:60:8 v:v), sonicated using both sonicators and centrifuged. All resulting supernatants were combined and evaporated overnight in a warm sand bath under a stream of air. Film left after evaporation was dissolved in C:M:H_20 (30:60:8 v:v) for saponification at pH11. Using 4M KOH, samples are kept at a pH of 11 for four hours at 50°. After four hours samples were allowed to cool to room temperature and were neutralized to pH7 using Glacial Acetic Acid and centrifuged at 3000rpm for 10 min. The supernatant was collected and the fatty acid pellet was washed with C:M:H_20 (10:10:1) and centrifuged a second time. The supernatants were combined and evaporated under a stream of air until only 500µl remain. The 500µl sample was passed through the G-50 sephadex quick spin size exclusion gel column (Roche, Canada) to further purify the sample. A DEAE Sepharose column (Amersham Bioscience, Canada) was prepared using washes of C:M:NaAcetate (0.8M) (30:60:8 v:v), C:M:H_2O (30:60:8 v:v). After several washes the sepharose was added to a column apparatus. The sample was passed through

the column followed by a wash with C:M:H_2O (30:60:8) to collect neutral glycolipids. Gangliosides were eluted by washing the column with C:M:NaAcetate (0.8M), all flow-throughs were combined and evaporated overnight under a stream of air. The evaporated film was re-suspended in C:M:KCL (0.1M) (3:48:47) and passed through a sep-PAK C-18 reverse phase column (Waters, WAT051910) prepared with washes of Methanol, C:M (2:1) and C:M:KCl (3:48:47), to elute the hydrophobic gangliosides and sialylated glycoconjugates. The final purified sample was re-suspended in C:M:H_2O (10:10:1) and stored at -20° in a sealed vial. Samples were dotted onto a silica Thin Layer Chromatography (TLC) plate and glycoconjugates were separated by charge and molecular weight using Acetone followed by C:M:$CaCl_2$ (0.2%) as the mobile phase buffer. After allowing the plate to dry a mixture of Resorcinol and H_2SO_4 was used to stain the resolved gangliosides. The stained plate was covered and heated at 110° and images were captured using UV exposure as well as bright light. Sample loading was equalized using protein levels of the original lysate determined by Lowry assay (BioRad).

2.8 Western Protein Analysis

For confirmation of Sialidase protein stability and expression, fibroblasts were grown and treated with 0.6μM MG132, 0.6μM Celastrol or a combination of 0.6μM MG132 and 0.6μM Celastrol in DMEM for 72 hours. For experiments examining allele specific protein levels, infection with AdSial^{+}, AdSial^{R225P} or AdSial^{R341G} was performed before treatment. After drug treatment, cells were harvested into lysis buffer containing protease inhibitors (Roche #14791200) and sonicated at 20% pulse. 2× laemmeli sample buffer was added to the lysate and boiled for 5 minutes before storage at -20°. Boiled samples were equally loaded into a 10% SDS-polyacrylimide (BioRad) gel and transferred at 120V for 70 minutes to a nitrocellulose membrane. Membranes were blocked using 5% non-fat milk (Carnation) in 1× TBST before probing with antibody probes, suspended in 5% milk in TBST, overnight

at 4°. After washing in 1× TBST five times, membranes were blotted with secondary IgG-HRP conjugated antibodies in 5% Milk in TBST (1:10 000), for one hour at room temperature followed by five washes in 1× TBST. Blotted membranes were incubated with ECL-chemiluminescent reagents (Amersham/GE) and exposed on Kodac X-ray film. Recombinant Sialidase was detected with an anti-polyhistidine antibody (1:1000)[48] and a goat anti-mouse IgG-HRP. Sialidase was probed for using the monoclonal human Sialidase antibody (1:1000) and goat anti-rabbit IgG-HRP (1:10 000). Grp78 was blotted for using the grp78 (N-20) antibody (1:1000) along with the donkey anti-goat IgG-HRP. Loading was normalized using a probe for β-Actin (1:1000) and the goat anti-mouse IgG-HRP. In instances where Actin was not a viable loading control Ponceau red staining was employed. Protein levels were analyzed using the Lowry assay (BioRad). Membranes were stripped of secondary antibodies between blotting using a stripping buffer at a pH of 2.2 composed of 7.5g glycine, 0.6g SDS, and 5ml Tween 20 in 500ml.

2.9 Quantification of Western Blot Analysis

X-ray films were scanned and saved as .JPEG files and analyzed using ImageJ (McMaster Biophotonics) software. Image colour was inverted and background was subtracted at 50 pixels. Using the free hand tool, bands were outlined and the area, mean density and integrated density was calculated in ImageJ. The integrated densities of each band were divided by the corresponding value for Actin and represent normalized protein levels.

2.10 Sialidase Enzyme Activity Analysis

Sialidase activity was examined in fibroblasts grown in 6 well plates. For specific activity of mutant Sialidase alleles, Sialidase-null cells were infected with AdSial^{+}, AdSial^{R225P} or AdSial^{R341G} prior to treatment. For dose dependant response experiments, Sialidosis fibroblasts were treated with concentrations of MG132 and Celastrol ranging from 0µM to 1.2µM.

All subsequent experiments used treatments of 0.6μM MG132, 0.6μM Celastrol or a both 0.6μM MG132 and 0.6μM Celastrol suspended in DMEM for 72 hours. Treated cells were washed two times in cold 1× PBS, scraped into distilled water and sonicated at 20% pulse. 50μl of cell lysate was added to a 60μl mixture of the artificial substrate (pH 4.2) (25μl) MuNANA, (10μl) BSA and (25μl) 0.8M NaAcetate buffer and incubated for 60min at 37°. The release of the artificial substrate MuNANA was stopped using 1.9ml of 0.1M MAP at a pH of 9.6. The fluorescence of 200μl of reaction substrate was measured using a Perkin Elmer LS Reader plate fluorometer at 355nm/460nm. Standard curves using known concentrations of Umbelliforone were used to assess Sialidase activity from MuNANA fluorescence readings in both white and black 96 well plates. Protein levels were determined by Bradford assay (BioRad). Units of specific activity (U) are representative of μMol of umbelliferone released per minute per mg of protein. Measurement of normal Sialidase enzyme activity using MCH64 cells was performed by Nicole Cholewinski and included for reference in the analysis of enzyme activity in Sialidosis cells.

Chapter 3

Results

3.1 Specific enzyme activity of endogenous Sialidase in human Sialidosis fibroblasts treated with MG132, Celastrol or MG132 and Celastrol in combination.

Human Sialidosis fibroblasts, heterozygous for the c.1021C>G (p.R341G) and c.3G>A (p.M1?) mutations of Neu1 were treated with increasing concentrations of either MG132 or Celastrol to establish treatment effect on Sialidase enzyme activity. A dose dependent increase in Sialidase activity is observed in fibroblasts treated with MG132 (Fig. 3.1). Enzyme activity in cells treated with 1.2μM MG132 increases almost five times above that of the no treatment control. An increasing trend was seen in cells treated with 0.6μM and 0.8μM MG132 with a near significant increase in specific activity of Sialidase after treatment with 1.0μM MG132 in comparison to the untreated control. In addition, fibroblasts treated with a concentration of 0.8μM and above show a considerable loss of cell number in culture corresponding with a threefold decrease in protein levels.

In a previous study, it has been shown that the positive effect of proteosomal inhibition on enzyme activity can be enhanced with the addition of certain chaperones and other proteosomal regulators like Celastrol[81]. Having established the dose dependent effect of MG132 on Sialidosis fibroblasts (Fig. 3.1), the effect of increasing concentrations of Celastrol on the specific activity of Sialidase in human Sialidosis cells is assessed. Fibroblasts treated with 0μM, 0.6μM or 0.8μM Celastrol for 72 hours (Fig. 3.2) are analysed and after treatment with 0.6μM Celastrol cells produce an almost 3 fold increase in Sialidase specific enzyme activity when compared to the no treatment control. Treatment with 0.8μM Celastrol produces no increase in activity indicating that increasing concentrations beyond 0.6μM does not have a positive effect on Sialidase activity. Interestingly, there are no observable changes in cellular viability with Celastrol treatment. Hence, all subsequent experiments conducted in our study used concentrations of 0.6μM MG132 and 0.6μM Celastrol to optimize effects

on enzyme activity while maintaining cell viability.

Sialidosis fibroblasts are utilized to examine the prospect that proteosomal inhibition and chaperone use can recover enzyme activity of defective Sialidase in vitro. In comparison to the no treatment control, treatment with MG132 alone does display an increasing trend in endogenous enzyme activity that is not statistically significant. Although Celastrol alone appears to have no effect on enzyme activity, when Sialidosis fibroblasts are treated with the combination of both MG132 and Celastrol, there is a significant increase in Sialidase enzyme specific activity ($p<0.001$) in comparison to no treatment control (Fig. 3.3). As a benchmark, the specific activity of Sialidase was measured in normal human fibroblasts (MCH64) and is included as a reference for normal Sialidase enzyme activity in fibroblasts.

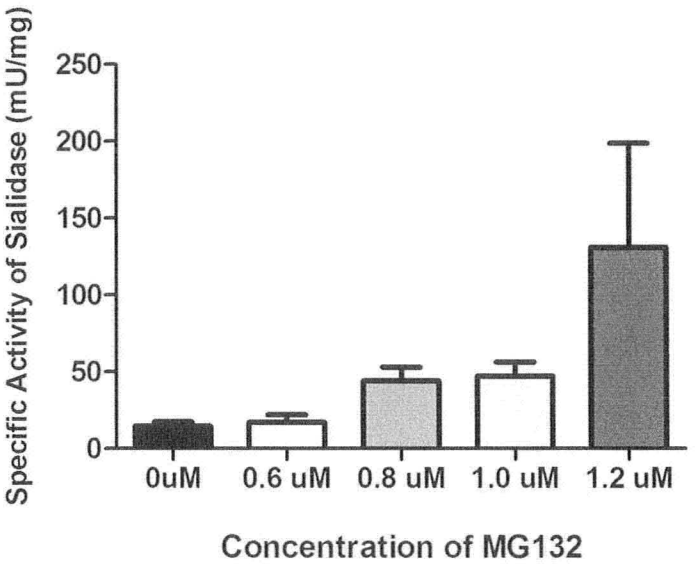


Figure 3.1: **Sialidase activity of Sialidosis cells in response to increasing concentrations of the proteosomal inhibitor MG132.** Human Sialidosis cells were treated with MG132 in DMEM for 72 hours at varying concentrations and assayed for enzymatic activity using the artificial substrate MuNANA at pH 4.2. Units of specific activity (U) is representative of μMol of umbelliferone released per minute per mg of protein. A dose dependent response is observed with specific activity doubling at 0.8μM and 1.2μM MG132. Tests of significance were performed using a one-way Anova followed by tukey's multiple comparison test. All error bars represent SEM where n=4 for 0μM and n=5 for 0.6μM to 1.2μM.

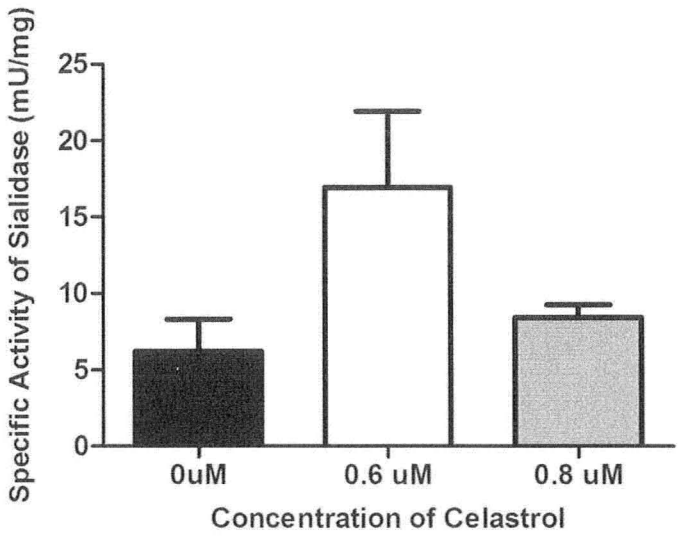


Figure 3.2: **Endogenous Sialidase activity of Sialidosis fibroblasts in response to increasing concentrations of the pharmacological chaperone Celastrol.** Human Sialidosis cells were treated with Celastrol in DMEM for 72 hours at varying concentrations and assayed for enzymatic activity using the artificial substrate MuNANA at pH4.2. Units of specific activity (U) are representative of μMol of umbelliferone released per minute per mg of protein. The only increase in Sialidase activity was seen after treatment of Sialidosis fibroblasts with 0.6μM Celastrol. Tests of significance were performed using a one-way Anova followed by tukey's multiple comparison test. All error bars represent SEM where n=4 for all samples.

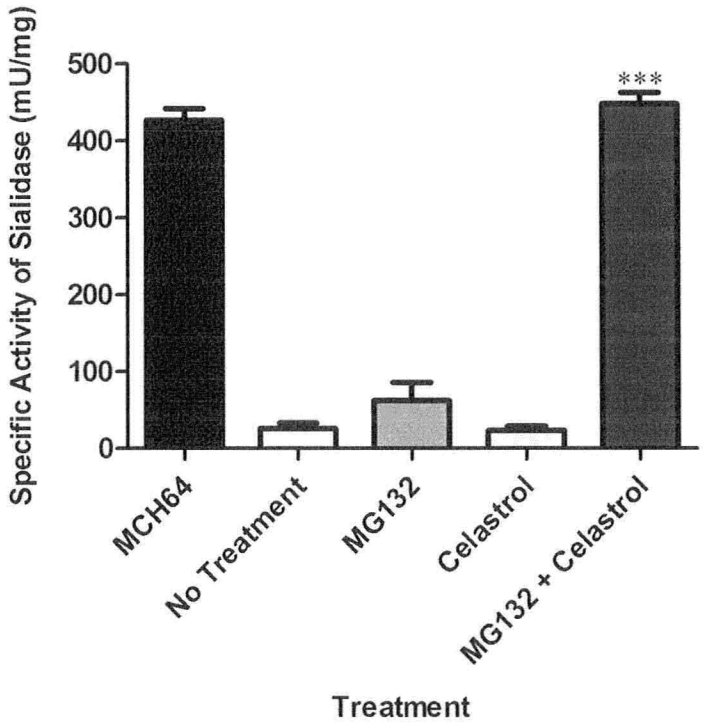
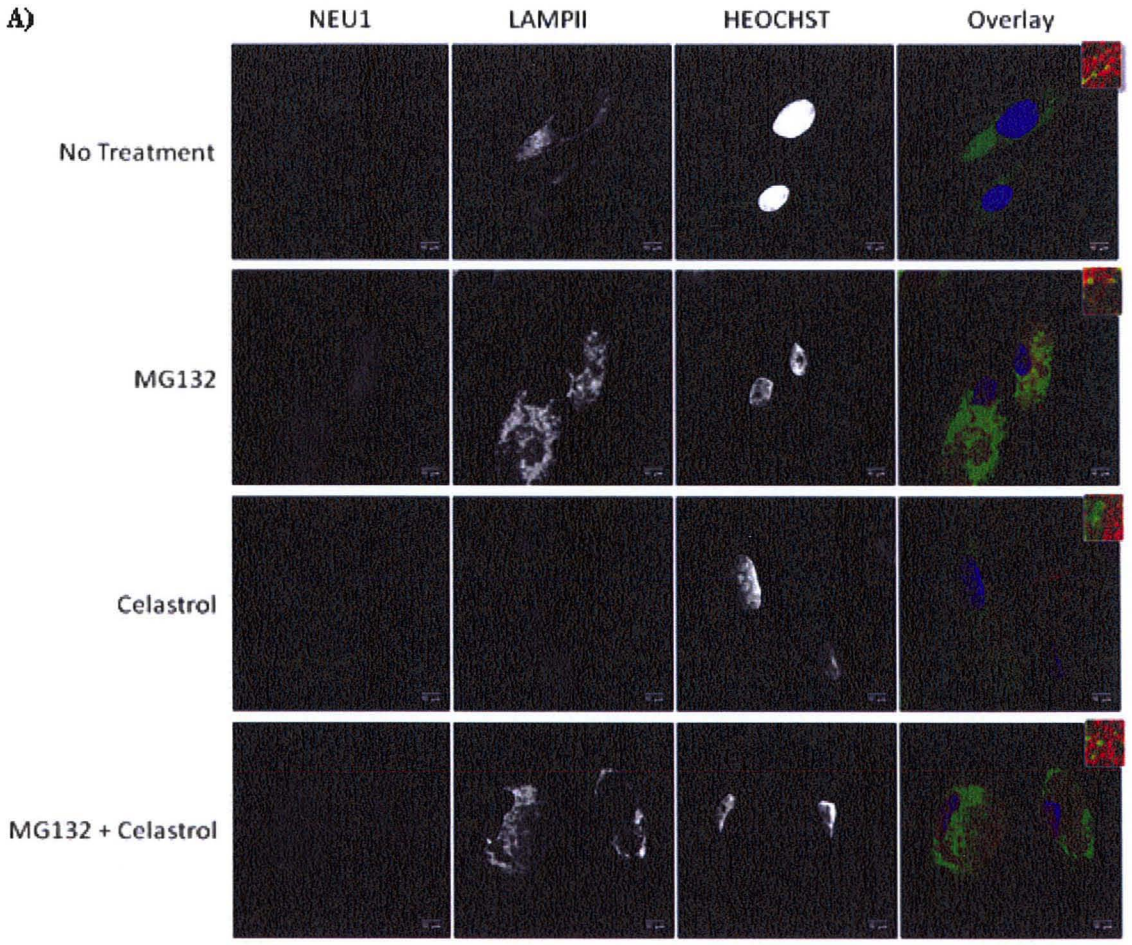


Figure 3.3: **Endogenous Sialidase activity of Sialidosis fibroblasts in response to MG132, Celastrol or a combination of both, MG132 and Celastrol.** Human Sialidosis cells were grown in 6 well plates and treated with 0.6μM MG132, 0.6μM Celastrol or a combination of both drugs at 0.6μM for 72 hours and assayed for enzymatic activity using the artificial substrate MuNANA at pH4.2. MCH64 cells were grown in 75cm^2 flasks and used as a baseline for normal Sialidase activity. Units of specific activity (U) are representative of μMol of umbelliferone released per minute per mg of protein. Combination of both MG132 and Celastrol resulted in a significant 17× increase in specific Sialidase activity. This increase seems to improve activity beyond that of the enzyme activity measured in the normal human fibroblasts (MCH64). No significant increase in activity is seen with the treatment of MG132 or Celastrol independently although MG132 does show an increasing trend. Tests of significance were performed using a one-way Anova followed by tukey's multiple comparison test. (***) indicates $p<0.001$ significance in comparison to the no treatment control. All error bars represent SEM where n=4 for all treatments of Sialidosis fibroblasts and n=3 for MCH64.

3.2 Intracellular localisation of endogenous Sialidase with the lysosomal marker LAMP II in human Sialidosis fibroblasts after treatment with MG132, Celastrol or both MG132 and Celastrol in combination

As shown previously, treatment with MG132 and Celastrol shows a significant increase in specific activity of the endogenous mutant Sialidase enzyme. In vivo, Sialidase is translated in the Endoplasmic Reticulum (ER) where it is folded and complexed with Protective Protein Cathepsin A. After further processing in the Golgi, Sialidase is transported to the lysosome where it hydrolyzes its endogenous substrates including sialylated carbohydrates and gangliosides[71,124]. It is well established that mutations in the Neu1 gene lead to misfolding of the protein which results in subsequent retention in the Endoplasmic Reticulum and impaired targeting to the lysosome[86]. Using immunofluorescent microscopy, we assessed the ability of MG132 and Celastrol to improve the proper targeting of Sialidase to the lysosome in Sialidosis fibroblasts. Using ant-Sialidase and anti-LAMP II, a lysosomal marker, the localisation of Sialidase within the lysosome was quantified after treatment with MG132 and Celastrol alone or in combination. The immunocytochemical staining of endogenous Sialidase is weak in untreated cells and staining across all treatments has a reticular network. A significant increase in Sialidase lysosomal localisation is evident in cells treated with MG132 and both MG132 and Celastrol, with no significant difference seen in those cells treated with Celastrol alone (Fig. 3.3A). Colocalisation of Sialidase with the lysosomal marker LAMP II is increased in cells treated with MG132 independently as well as in cells treated with the combination of MG132 and Celastrol. Mander's Coefficient is utilized to identify the degree of overlap between Sialdiase and LAMP II[139]. Mander's Coefficient gives a percentage of colour one (red) pixels that overlap with colour two (green) pixels in an image. This quantification revealed a significant increase in the colocalisation of

Sialidase with LAMP II in one optical slice of cells treated with MG132 ($p<0.01$) and cells treated with both MG132 and Celastrol ($p<0.05$) (Fig. 3.3B). Interestingly, there seems to be no additive increase in colocalisation of Sialidase with LAMP II in the presence of both drug treatments despite the additive increase in specific enzyme activity generated when cells are treated with both MG132 and Celastrol.


A)
NEU1
LAMPII
HEOCHST
Overlay
No Treatment
MG132
Celastrol
MG132 + Celastrol

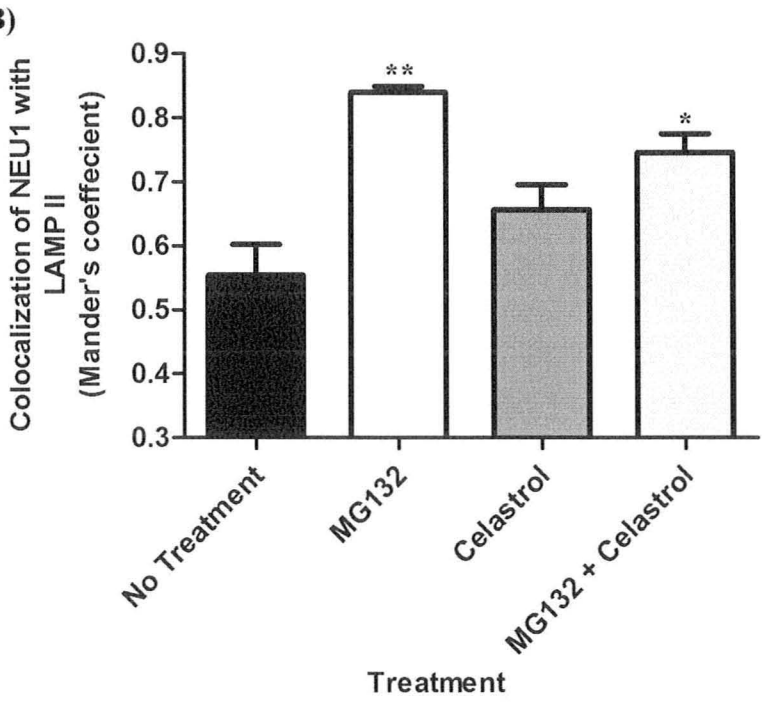


Figure 3.3: **Intracellular colocalization of endogenous Sialidase with the lysosomal marker LAMP II in response to treatment with MG132, Celastrol or both MG132 and Celastrol.** (A)Human Sialidosis fibroblasts were grown on cover slips and treated with, 0.6μM MG132, 0.6μM Celastrol or both 0.6μM MG132 and 0.6μM Celastrol in combination. Following 72 hours incubation, cells were stained with anti-Sialidase and anti-LAMP II as described previously[48]. Images were generated using the Leica LF software and are representative of the entire population of cells. An observable increase in Sialidase staining can be seen in cells treated with MG132 and the combination of MG132 and Celastrol. (B) Meander's coefficient quantification of red:green overlap was measured from one optical slice of cells from at least two fields of view on all slides and represents colocalisation of Sialidase with the lysosomal marker LAMP II. An increase in colocolisation of Sialidase with the lysosomal marker LAMP II is shown using Meander's coefficient in cells treated with both MG132 alone and MG132 and Celastrol in combination. (*) designates a significance of $p<0.05$ while (**) represents significance of $p<0.01$ using Anova followed by tukey's multiple comparison test for an n between three and eight. Error bars represent SEM. All images were acquired at 630× magnification.

3.3 Ganglioside levels in Sialidosis fibroblasts pre-loaded with gangliosides after treatment with MG132, Celastrol or both MG132 and Celastrol

To elucidate the impact of drug treatment on the Sialidosis fibroblasts ability to catabolise the natural ligand, gangliosides in vitro, fibroblasts were pre-loaded with 200μg of a mixture of bovine brain gangliosides and were treated with MG132, Celastrol or both. After isolation, separation, and detection of ganglioside metabolites, a significant depletion of the complex gangliosides, including the tri-sialo ganglioside band (GT1b) is observed in the samples treated with Celastrol alone or in combination with MG132 (Fig. 3.4). In fact, treatment of Sialidosis fibroblasts with MG132 drastically changes the banding pattern amongst the monosialo-glycocconjugates. Furthermore, there is a clear reduction of GD3, collapsing the doublet into one single band, as well as a depletion of gangliosides in the vicinity of the mono- and a-sialo gangliosides GM2 and GA2 indicating that gangliosides of similar weight and charge are being depleted in the presence of MG132. The glycosphingolipid accumulation pattern of the cells treated with both MG132 and Celastrol in combination shows the same reductions as cells treated with each pharmacological agent independently confirming a synergistic effect of treatment with both drugs (Fig. 3.4).

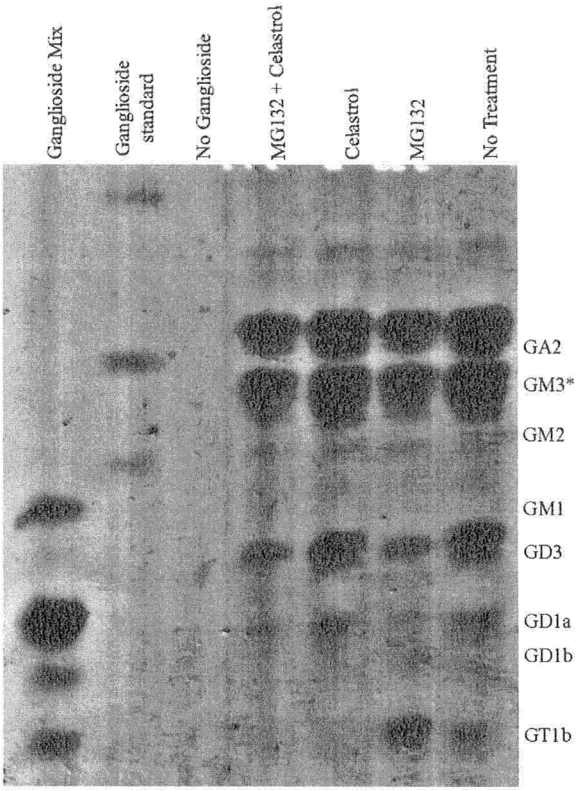


Figure 3.4: **TLC analysis of gangliosides and glycolipids isolated from human Sialidosis fibroblasts pre-loaded with gangliosides after treatment with MG132, Celastrol or both MG132 and Celastrol in combination.** Human Sialidosis fibroblasts were incubated in Opti-MEM containing 200µg of a ganglioside mixture for five days. Cells were then treated with MG132, Celastrol or both MG132 and Celastrol in combination for 72 hours. Cells were harvested and glycosphingolipids were isolated and seperated on a silica TLC plate. GM3* represents the putative location of GM3 on the TLC. Samples were normalized using equal amounts of protein in original cell extract. Gangliosides and glycolipids were stained using Resorcinol in H_2SO_4. Note the depletion of the higher complexity gangliosides including GT1b and GD1b in the presence of Celastrol. MG132 has effects on less complex gangliosides depleting GD3 and diminishing the bands surrounding the less complex GM2 and GA2. The additive effect of treatment with both MG132 and Celastrol can be seen from the dramatic depletion of the gangliosides which mirror that of the individual treatments.

3.4 Effects of MG132 and Celastrol on Sialidase activity in Sialidase-null fibroblasts infected with Adenoviruses carrying normal or mutant alleles of Neu1 cDNA

With over 40 known mutations of Neu1 leading to varying phenotypic severities of Sialidosis [70,105], we aim to assess if positive effects of MG132 and Celastrol treatment could be observed after the expression of specific alleles of Neu1 harbouring different mutations. To examine the effect of treatment on Sialidase mutant alleles, a null Sialidase cell line (WG0544) is utlilized along with adenovirus expressing mutant human Neu1 cDNAs. The specific mutations examined are the c.1021C>G (p.R341G) and c.674G>C (p.R225P) mutations[86]. Cells are infected with an adenovirus expressing normal ($AdSial^{+}$), R225P ($AdSial^{R225P}$) or R341G ($AdSial^{R341G}$) Sialidase. After 24h of infection, fibroblasts are treated with 0.6μM MG132, 0.6μM Celastrol or a combination of 0.6μM MG132 and 0.6μM Celastrol. The addition of MG132 significantly increases the enzyme specific activity of defective Sialidase enzyme harbouring the R225P and R341G substitutions (Fig. 3.5). In cells expressing the R225P or the R341G mutant Sialidase, enzyme activity displays a 2× and 7.5× fold increase respectively in response to MG132 when compared to no treatment control. Although Celastrol does not induce a significant increase in Sialidase activity, a trend toward increasing activity is observed in cells expressing either mutant alleles (Fig. 3.5). Treatment with both MG132 and Celastrol leads to a significant fourfold increase in Sialidase specific activity in both $AdSial^{R225P}$ and $AdSial^{R341G}$ infected cells in comparison to their no treatment controls. The synergistic effect of MG132 and Celastrol is confirmed in the R225P mutant allele expressing fibroblasts, as enzyme activity significantly improves even above activity in cells treated with MG132 alone. Cells expressing the R341G mutant allele however, show a an improvement of Sialidase activity but this increase does not overcome the effect of MG132 alone which approaches enzyme activity levels acquired by normal allele

expressing fibroblasts.

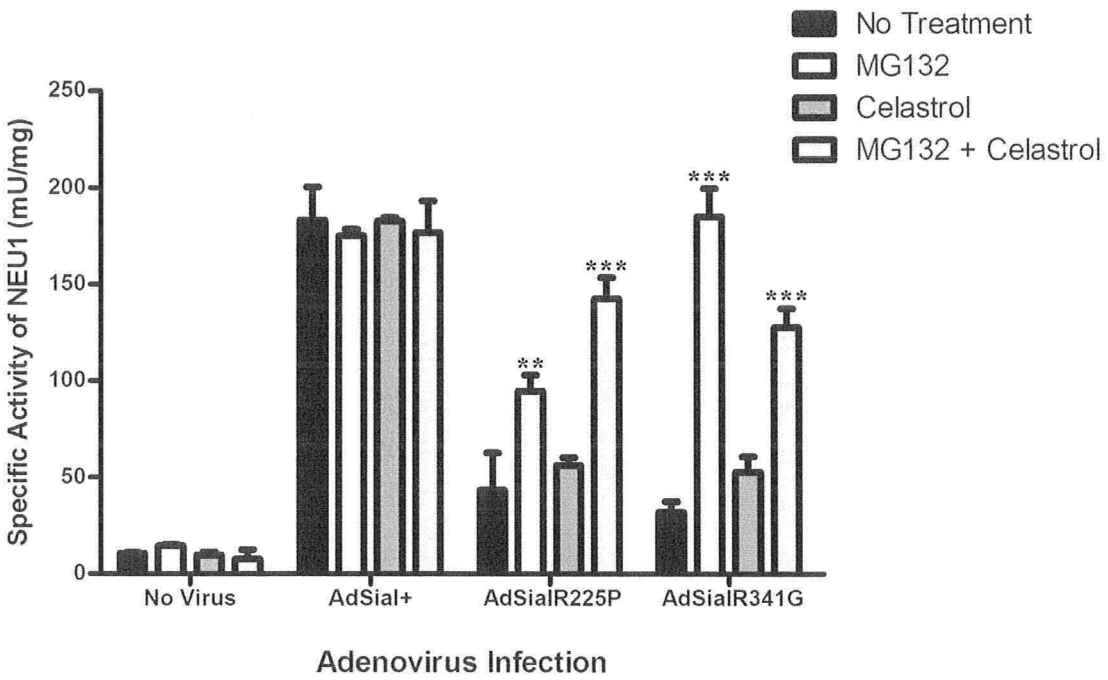


Figure 3.5: **Sialidase activity in human Sialidas-null fibroblasts expressing wild type or mutant alleles of Neu1 cDNA after treatment with MG132, Celastrol or MG132 and Celastrol in combination.** Sialidase-null fibroblasts were infected with AdSial^{+}, AdSial^{R225P} or AdSial^{R341G} expressing normal or mutant alleles of Neu1 cDNA. Cells were treated with 0.6μM MG132, 0.6μM Celastrol or a combination of both drugs at a concentration of 0.6μM and assayed for enzymatic activity. In both the AdSial^{R225P} and AdSial^{R341G} infected fibroblasts, treatment with MG132 dramatically increases the specific enzyme activity. Fibroblasts expressing the R341G mutant Neu1 cDNA treated with MG132 show a recovery of enzyme activity to levels equal to activity in cells expressing the normal allele. In the AdSial^{R225P} infected cells, the combination of MG132 and Celastrol is more effective than treatment with MG132 alone. However, in cells infected with AdSial^{R341G} a positive though not additive effect is achieved upon treatment with both MG132 and Celastrol. Units of specific activity (U) are representative of μMol of umbelliferone released per minute per mg of protein. A two way anova followed by Bonferroni test of significance was performed for each virus group. (**) signifies $p<0.01$ significance in comparison to the no treatment control in each adenovirus group, (***) indicates $p<0.001$ significance in comparison to the no treatment control. All error bars represent SEM where n is between three and four replicates of each treatment.

3.5 Effects of MG132 and Celastrol treatment on levels of protein expression in human Sialidase-null fibroblasts infected with adenovirus expression normal and mutant Sialidase cDNA

Western Blot analysis was used to determine the effects of drug treatment on steady state expression of Sialidase in cells infected with AdSial^{+}, AdSial^{R225P} and AdSial^{R341G}. To assess the protein expression of fibroblasts expressing mutant alleles post treatment, we utilized two anti-Sialidase antibodies: one, anti-Sialidase, recognizes the carboxyl (COOH) terminal of the protein while the second, anti-RGS His, recognizes the poly Histidine tag at the amino (NH2) terminal of the transiently expressed recombinant Sialidase[47].

Typically, human Sialidase has 3 conserved N-glycosylation sites[128] resulting in 3 visible bands at 45, 47, and 49kDa[86]. Our results reveal similar molecular species of Sialidase in cells expressing either mutant allele as well as those expressing the normal allele of Sialidase cDNA (Fig. 3.6 - 3.8). In cells expressing the c.674G>C(p.R225P) mutant allele cDNA, there are observable changes in steady state levels of Sialidase after drug treatment (Fig. 3.6A). Lysates blotted for Sialidase using the anti-COOH Sialidase antibody shows that treatment with MG132 causes an increase in the level of detectable Sialidase, while the addition of Celastrol seems to diminish Sialidase levels (Fig. 3.6B). No additive effect on steady state Sialidase expression is observed after treatment with both MG132 and Celastrol. Fibroblasts expressing mutant allele cDNA treated with MG132 display a 15 kDa protein when blotted with anti-COOH Sialidase antibody; this 15 kDa Sialidase positive protein is not visible after blotting with anti-RGS His (Fig. 3.6A). As anti-COOH antibody recognizes the carboxyl terminal of Sialidase, the 15 kDa protein corresponds to a cleaved C-terminal portion of Sialidase. Treatment of cells with N-glycosidase F does not affect this fragment confirming its lack of glycosylation sites. Similar protein expression is observed in

cells infected with the AdSial^{R341G} expressing the c.1021C>G (p.R341G) Sialidase mutant allele cDNA (Fig. 3.7A). As seen in the cells expressing the c.674G>C (p.R225P) mutant Sialidase, treatment with MG132 increases protein expression that of no treatment control, while Celastrol reduces Sialidase expression. Also, blotting membranes with anti-COOH Sialidase antibody reveals the 15 kDa species that is not revealed using anti-RGS His. There is also an additive increase in steady state Sialidase seen in the cells infected with AdSial^{R341G} and treated with both MG132 and Celastrol (Fig. 3.7B).

Cells infected with AdSial^{+}, expressing the normal Sialidase allele, produces higher Sialdiase expression after MG132 treatment (Fig. 3.8A). However, there is no additive effect in cells treated with both MG132 and Celastrol. In contrast, treatment with Celastrol alone does drastically diminish the level of steady state Sialidase (Fig. 3.8B).

To determine the effects of drug treatment on N-glycosylation of Sialidase, cell samples were incubated with N-glycosidase F which cleaves N-linked oligosaccharides. There is a decrease in molecular size after incubation of cell lysates with N-glycosidase F. All 3 isoforms of Sialidase, at 45, 47 and 49 kDa are present in lysates with no N-glycosidase F treatment. However, there is a shift in molecular weight of all three isoforms of Sialidase when lysates are treated with N-glycosidase F (Fig. 3.6A, Fig. 3.7B). The addition of MG132 allows the various isoforms to resolve more without N-glycosidase F treatment. Celastrol does treatment produces no change in isoform molecular weights.

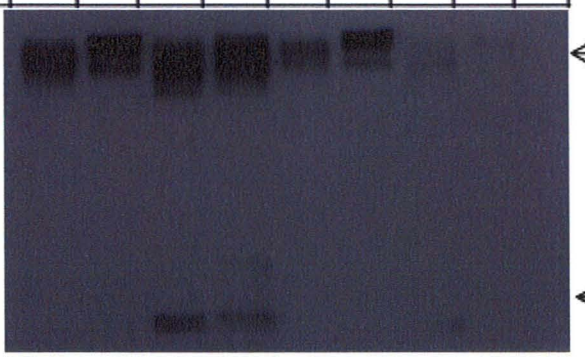
Anti-RGS His antibody recognizes a poly-histidine tag inserted in the NH2 terminal end of Sialidase[47]. Using this antibody, we confirm which accumulations of Sialidase levels were consistent with both the NH2 and COOH terminals. What we observe is that with all mutant allels as well as with normal alleles we see an increase in Sialidase levels with treatment of MG132, although this increase is more defined in cells expressing the R225P mutant allele versus those expressing the R341G mutant allele. In cells expressing normal alleles of Sialidase, there are drastic increases in anti-NH2 antibody positive Sialidase after treatment with MG132. In cells expressing either mutant allele or normal Sialidase, a large

increase in Sialidase levels is observed after probing lysates with the anti-RGS His Sialidase antibody (Fig. 3.6 - 3.8). The small 15 kDa isoform of Sialidase was not present in lysates blotted with the anti-RGS His Sialidase antibody, confirming that this species is located at the C-terminus of Sialidase and not near the N-terminus.

Grp78 (BiP) is a chaperone that is upregulated during activation of Endoplasmic Reticulum's Associated Degredation (ERAD)[30]. To examine the affect of MG132 and Celastrol treatment on this commonly associated ERAD protein expression, the regulation of Grp78 is examined in cells expressing mutant allele Neu1 cDNA. Lysates were blotted for anti-Grp78 (N-20) to clarify the effects of treatment on cells expressing specific Sialidase mutations. We see that there are two isoforms of Grp78 in all cells and that the lower molecular weight isoform is increased in the presence of MG132 while the larger molecular weight remains the same. This effect is observed in cells expressing either mutant cDNA or normal Sialdiase(Fig. 3.6 - Fig. 3.8). Celastrol treatment results in no change in the intensity of the higher molecular weight bands from no treatment controls.

A)

N- Glycosidase F	+	-	+	-	+	-	+	-	-
MG-132	-	-	+	+	-	-	+	+	-
Celastrol	-	-	-	-	+	+	+	+	-
AdSial^{R225P} Virus	+	+	+	+	+	+	+	+	-



B)

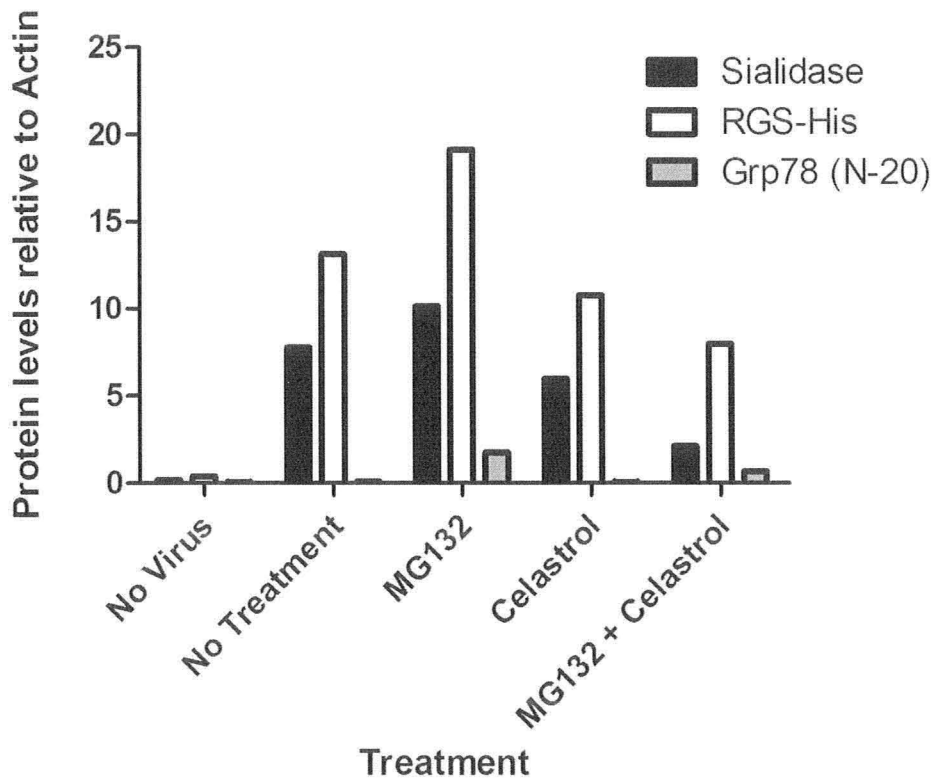
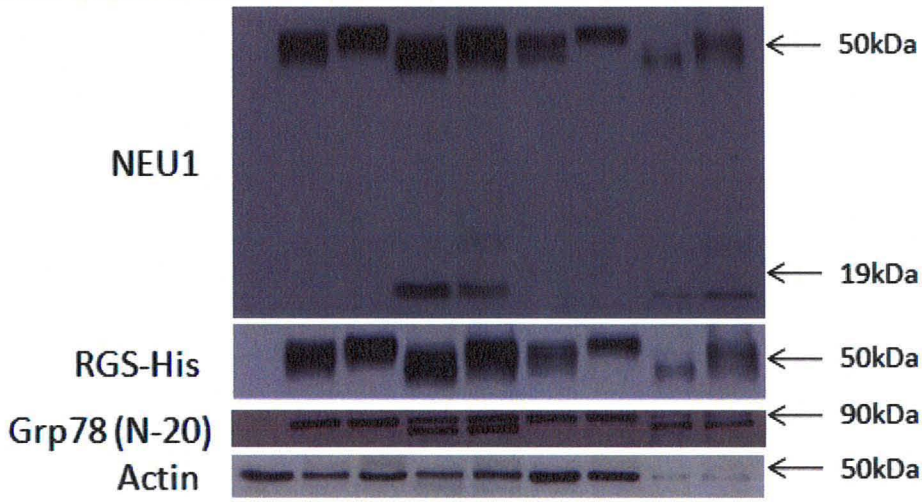

Protein levels relative to Actin
25
20
15
10
5
0
Sialidase
RGS-His
Grp78 (N-20)
No Virus
No Treatment
MG132
Celastrol
MG132 + Celastrol
Treatment

Figure 3.6: **Western blot analysis of human Sialidase-null fibroblasts infected with adenovirus carrying mutant Neu1 cDNA with the c.674G>C (p.R225P) substitution.** A) Human Sialidase-null cells infected with AdSial^{R225P} and treated with MG132, Celastrol or both MG132 and Celastrol followed by treatment with N-glycosidase F or no treatment. Membranes were probed using the anti-COOH Sialidase antibody and the goat anti-rabbit IgG HRP, anti-NH2 poly-histidine antibody along with goat anti-mouse IgG HRP and the anti-Grp78 (N-20) antibody and donkey anti-goat IgG HRP. Equal loading was confirmed by probing membranes for Actin using anti-Actin antibody and goat anti-mouse IgG HRP. With the addition of N-glycosidase F there is a decrease in molecular size of all Sialidase positive bands independent of drug treatment, however, heterogeneity of banding is maintaine. A small 15 kDa species unaffected by N-glycosidase F treatment appears when cells are treated with MG132. RGS-His blotting confirms Sialidase as a product of the recombinant transient expression of Sialidase. RGS-His expression mirrors that of anti-COOH Sialidase positive bands, except the 15kDa species seen in cells treated with MG132. B) Densitometry analysis of immunoblots was performed using ImageJ software. All bands were normalized using the intensity of its Actin counterpart. Quantification is representative of expression with no N-glycosidase F treatment. Quantification of Sialidase and Grp78 positive bands show that with treatment of MG 132 alone there is an increase in the Sialidase levels of expression over no treatment control, in contrast there is a decrease in Sialidase expression levels after Celastrol treatment. The combination of both drugs does not have an additive effect on Sialidase expression levels in cells infected with AdSial^{R225P}. Expression of the ERAD indicator Grp78 also increases with MG132 expression while Celastrol alone imparts no change in Grp 78 expression levels. The combination of both MG132 and Celastrol does not have an additive effect above that of MG132 alone.

A)

N- Glycosidase F	+	+	-	+	-	+	-	+	-
MG-132	-	-	-	+	+	-	-	+	+
Celastrol	-	-	-	-	-	+	+	+	+
AdSial^{R341G} Virus	-	+	+	+	+	+	+	+	+



B)

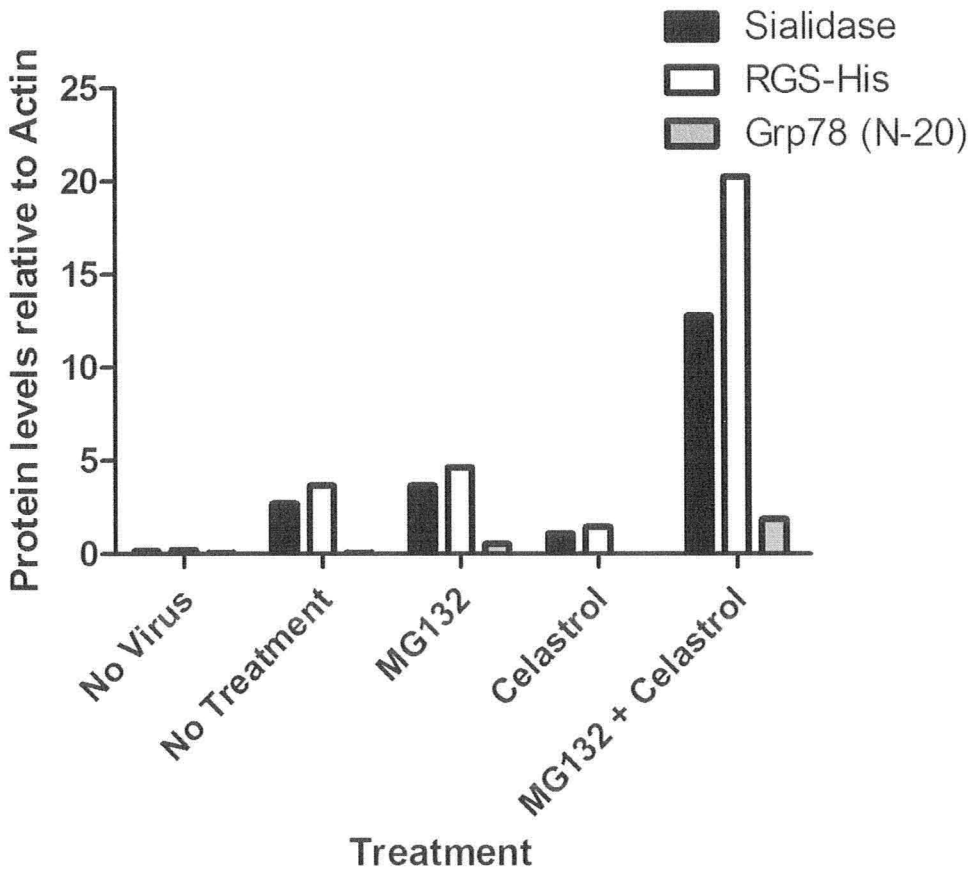
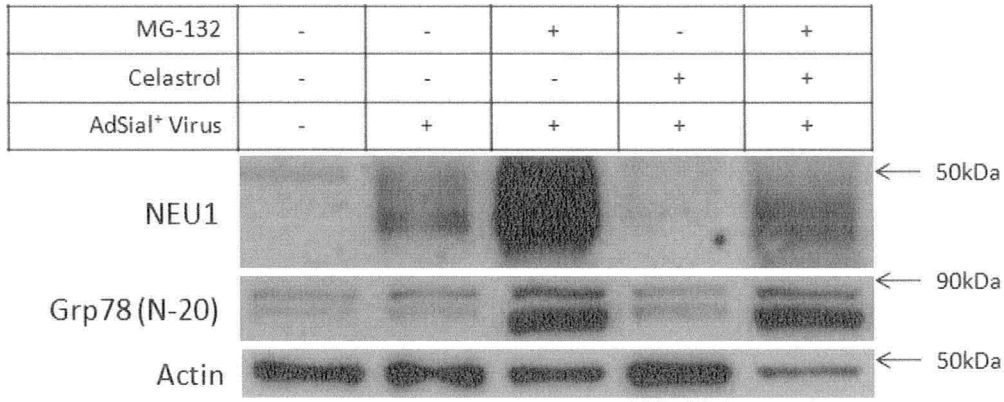

Sialidase
RGS-His
Grp78 (N-20)
Protein levels relative to Actin
25
20
15
10
5
0
No Virus
No Treatment
MG132
Celastrol
MG132 + Celastrol
Treatment

Figure 3.7: **Western blot analysis of human Sialidase-null fibroblasts infected with adenovirus carrying mutant Neu1 cDNA with the c.1021C>G (p.R341G) substitution.** A) Human Sialidase-null cells infected with AdSial^{R341G} and treated with MG132, Celastrol or both MG132 and Celastrol followed by treatment with N-glycosidase F or no treatment. Membranes were probed using the anti-COOH Sialidase antibody and the goat anti-rabbit IgG HRP, anti-NH2 poly-histidine antibody along with goat anti-mouse IgG HRP and the anti-Grp78 (N-20) antibody and donkey anti-goat IgG HRP. Equal loading was confirmed by probing membranes for Actin using anti-Actin antibody and goat anti-mouse IgG HRP. With the addition of N-glycosidase F there is a decrease in molecular size of all Sialidase positive bands independent of drug treatment, however, heterogeneity of banding is maintaine. A small 15 kDa species unaffected by N-glycosidase F treatment appears when cells are treated with MG132. RGS-His blotting confirms Sialidase as a product of the recombinant transient expression of Sialidase. RGS-His expression mirrors that of anti-COOH Sialidase positive bands, except the 15kDa species seen in cells treated with MG132. Lysates treated with N-glycosidase F show a decrease in molecular size of all visible isoforms, with a more dramatic decrease in molecular size of the Sialidase after treatment with MG132 and Celastrol. There is an observable collapsing of the three isoforms of Sialdiase after treatment with both MG132 and Celastrol, however this is not observed after any other treatments. The novel 15 kDa C-terminal positive species of Sialidase is visible in lysates that have been treated with MG132 alone or in combination with Celastrol. This 15kDa species is not visible after blotting with anti-NH2 RGS-His antibody. B) Densitometry analysis of immunoblots was performed using ImageJ software. All bands were normalized using the intensity of its Actin counterpart. Quantification is representative of expression with no N-glycosidase F treatment. Quantification of Sialidase expression does increase with the treatment of MG132 with a dramatic increase in expression levels after treatment with both MG132 and Celastrol. Celastrol diminishes the expression levels of both anti-COOH Sialidase positive and anti-NH2 RGS-His positive species of Sialidase. There is an increase in expression levels of the ERAD initiated chaperone Grp78 is increased in cells treated with MG132 alone or in combination.

A)


MG-132 - - + - +
Celastrol - - - + +
AdSial+ Virus - + + + +
NEU1
Grp78 (N-20)
Actin
50kDa
90kDa
50kDa

B)

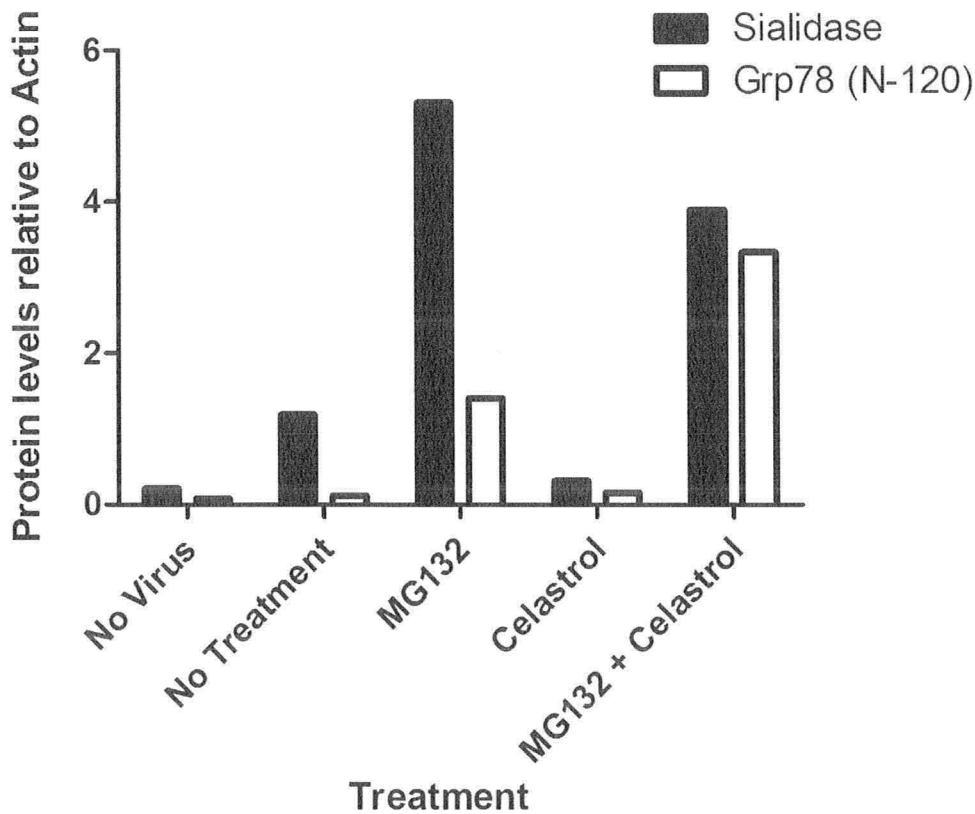

Sialidase
Grp78 (N-120)
Protein levels relative to Actin
0
2
4
6
No Virus
No Treatment
MG132
Celastrol
MG132 + Celastrol
Treatment

Figure 3.8: **Western blot analysis of human Sialidase- null fibroblasts infected with adenovirus carrying the normal human Neu1 cDNA after treatment with MG132, Celastrol or MG132 and Celastrol in combination.** A) Human Sialidase-null cells infected with AdSial^{+} and treated with MG132, Celastrol or both MG132 and Celastrol followed by treatment with N-glycosidase F or no treatment. Membranes were probed using the anti-COOH Sialidase antibody and the goat anti-rabbit IgG HRP, anti-NH2 poly-histidine antibody along with goat anti-mouse IgG HRP and the anti-Grp78 (N-20) antibody and donkey anti-goat IgG HRP. Equal loading was confirmed by probing membranes for Actin using anti-Actin antibody and goat anti-mouse IgG HRP. There is a an abundant amount of Sialidase in cells infected with the AdSial^{+} virus in comparison to the no virus control, particularly cells treated with MG132 alone. Cells treated with Celastrol have a diminished Sialidase expression. B) Densitometry analysis of immunoblots was performed using ImageJ software. All bands were normalized using the intensity of its Actin counterpart. Treatment with MG132 alone results in a 3X increase in Sialidase expression levels, while cells treated with both MG132 and Celastrol also produces an increase in expression level but not surpassing that seen after treatment with MG132 alone. Celastrol diminishes the expression levels of Sialidase while the expression of the ERAD initiated chaperone Grp78 is increased in cells treated with MG132 alone and more significantly in cells treated with both MG132 and Celastrol.

3.6 Intracellular localisation of Sialidase to the Lysosomal marker LAMP II in human Sialidase-null cells expressing normal and mutant alleles of Sialidase cDNAs after treatment with MG132, Celastrol or both MG132 and Celastrol

Once the drug effects on Sialidase enzyme activity and expression were established it is necessary to now determine if Sialidase is being appropriately targeted to the Lysosome in cells treated with MG132 and Celastrol. To assess targeting, cells were grown on round cover slips and infected with AdSial^{+} (MOI 100), AdSial^{R225P} (MOI 10) or AdSial^{R341G} (MOI 10) followed by drug treatment for 72 hours. Co-staining was performed as described previously[48] with anti-Sialidase and anti-LAMP II, a lysosomal marker, followed by staining with the anti-rabbit AlexaFluor 594 and anti-mouse AlexaFluor 488 to visualise LAMP II and Sialidase respectively.

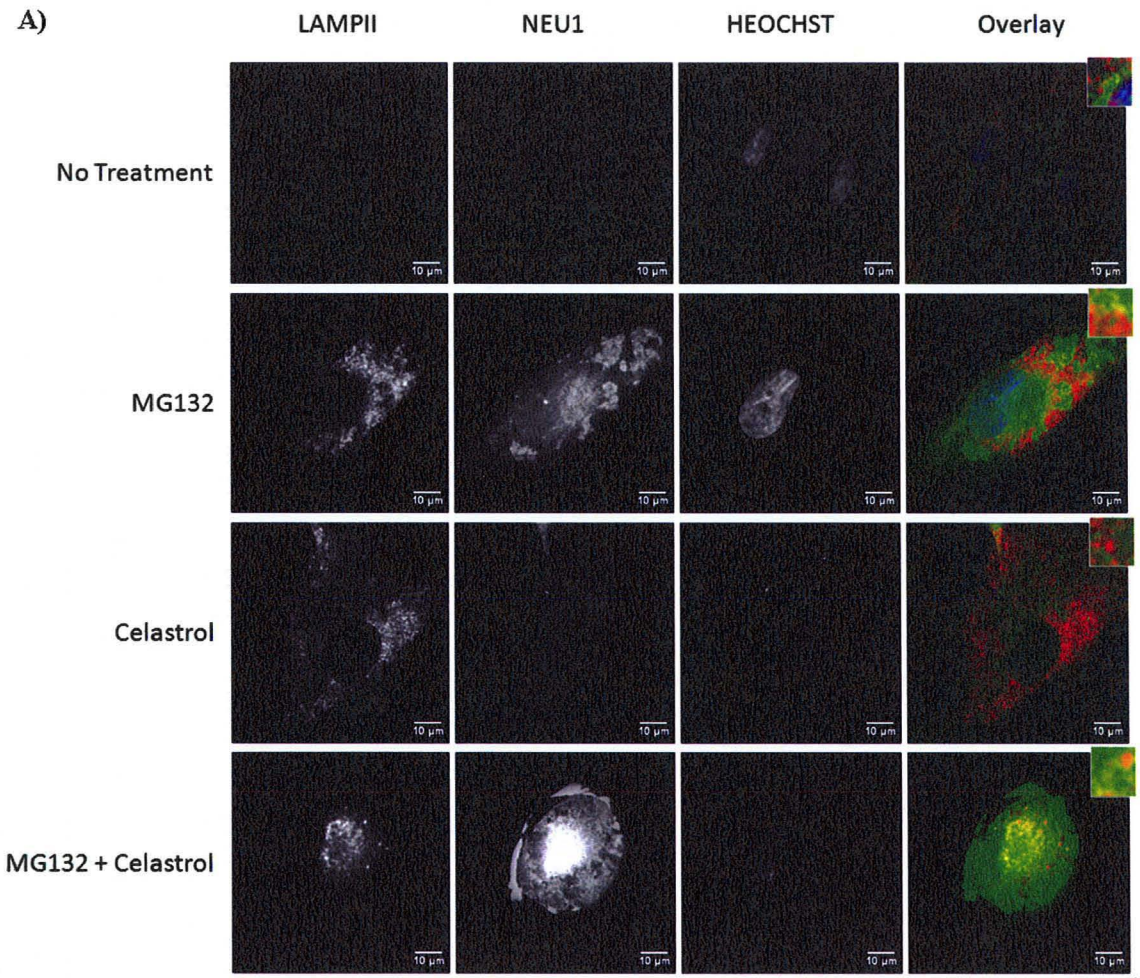
In general, immunocytochemical staining of cells infected with recombinant adenovirus expressing mutant alleles and normal Sialidase displays increased Sialidase expression after treatment with MG132 (Fig. 3.9A, 3.10A, 3.11A). In untreated cells infected with AdSial^{R225P}, the Sialidase staining resembles a reticular network without clear punctate formation. After treatment with MG132, cells develop a globular staining as well as organized punctate staining. Similar staining is observed in cells expressing the R225P mutant Sialidase that are treated with both MG132 and Celastrol. In comparison to no treatment control, treatment with Celastrol leads to an increase in the amount of Sialidase staining, with a modest increase in punctate formation (Fig. 3.9A). Cells infected with AdSial^{R341G} display a more organized punctate staining of Sialidase in no treatment control, in the MG132 treated cells as well as in cells treated with a combination of both MG132 and

Celastrol. Celastrol does not produce changes in Sialidase staining when used independently on cells expressing mutant alleles of Sialidase cDNA. In fact, a minor increase in Sialidase staining with Celastrol treatment with no distinctive increases in punctate staining, nor colocalisation with the lysosomal marker LAMP II (Fig. 3.10A). After infection with AdSial^{+}, Sialidase staining displays punctate formations with little reticular staining in all treatments. After treatment with MG132, there is an increase in the amount of Sialidase staining and subsequent increase in colocalisation with the lysosomal marker, LAMP II.

The effect of drug treatment on cells was not limited to enzyme localisation but also affected the size of cells when treated with MG132. Morphological changes in cells included cells becoming rounder and longer after treatment with MG132. Using the Zeiss inverted fluorescent microscope, images of nuclei were acquired for quantification of nuclear size. Measurement revealed an increase in cell size in those fibroblasts expressing both wild type (Fig. 3.12A), and mutant R225P Sialidase (Fig. 3.12B) when treated with MG132. In fibroblasts that have recombinant mutant R341G Sialidase expression, there is an increase in nuclear size after treatment with MG132 as well as with Celastrol independently (Fig. 3.12C).

Using Mander's Coefficient we quantified the colocalisation of Sialidase with the lysosomal marker LAMP II. Treatment of cells expressing either the R225P or R341G mutant Sialidase with MG132 produces a significant ($p<0.001$) increase in subcellular localisation of Sialidase with the lysosomal marker LAMP II (Fig. 3.9B, 3.10B). MG132 increases colocalisation of Sialidase with LAMP II in cells expressing the mutant alleles R225P and R341G by four times and three times respectively above that of no treatment control (Fig. 3.9B, Fig. 3.9B). Similar increases in intracellular localisation of Sialidase with the lysosome are seen in cells treated with the combination of both MG132 and Celastrol (a threefold increase) (Fig. 3.9B, 3.10B). Of note, there is no additive effect seen after treatment of cells with the combination of MG132 and Celastrol over cells treated with MG132 alone when examining colocalisation of Sialidase with the lysosomal marker. In cells expressing either

normal or mutant Neu1 cDNA. Celastrol has a limited effect on colocalisation with only the cells expressing the R341G mutant Sialidase showing an increase in colocalisation (Fig. 3.10B). In fibroblasts infected with the AdSial^{+} a similar pattern of increased localisation of Sialidase with the lysosome after treatment with MG132 is evident (fig. 3.11B).


A)
LAMPII
NEU1
HEOCHST
Overlay
No Treatment
MG132
Celastrol
MG132 + Celastrol
10 µm

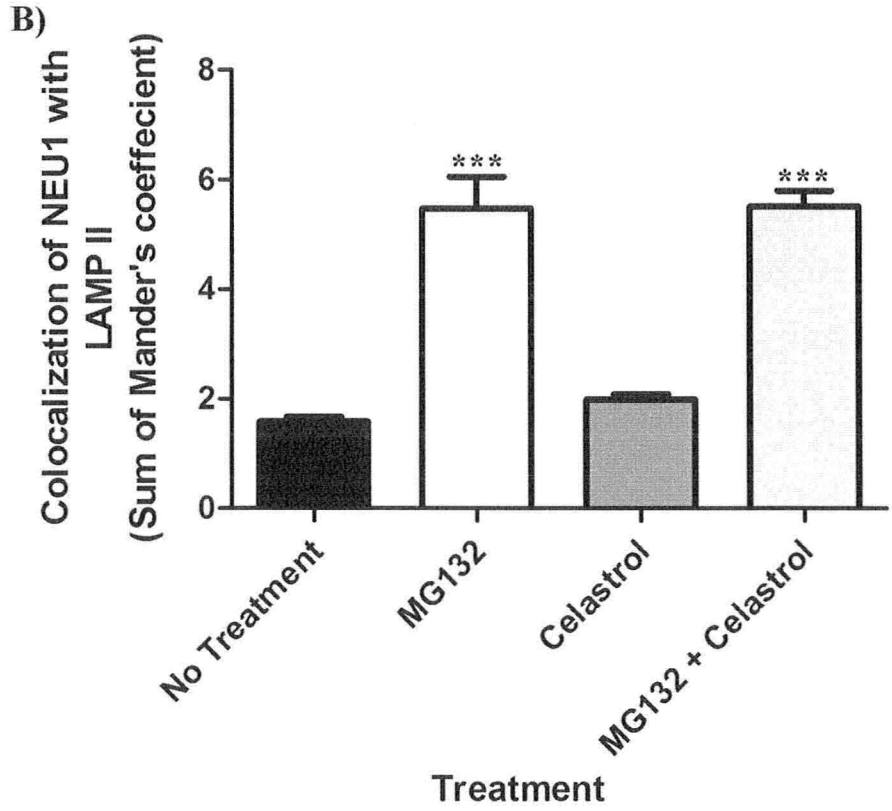
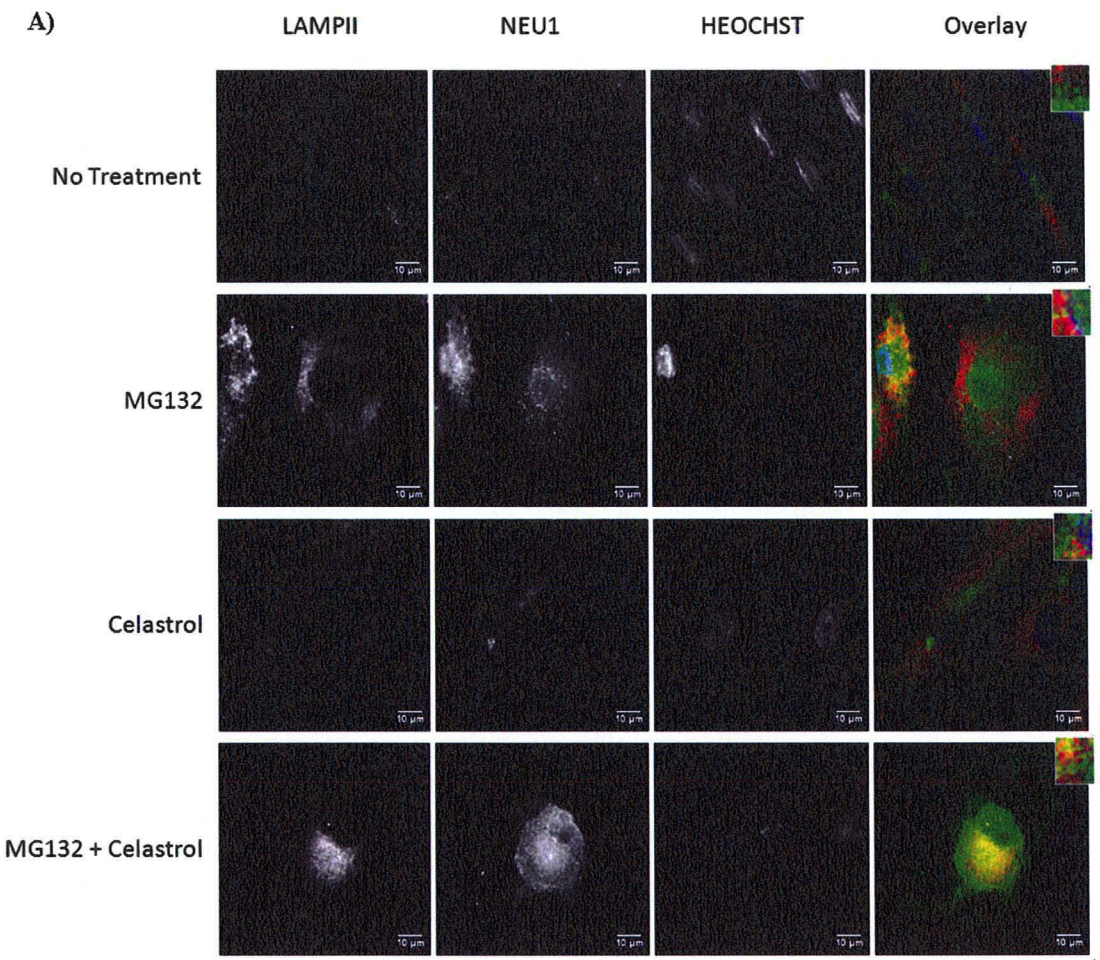


Figure 3.9: **Assessment of the intracellular co-localisation of Sialidase with the lysosomal marker LAMP II in Sialidase-null fibroblasts expressing the mutant R225P Sialidase.**(A) Human Sialidase-null fibroblasts infected with AdSial^{R225P} were treated with MG132, Celastrol or a combination of both MG132 and Celastrol, fixed, permeabolized and immunostained for Sialidase and LAMP II. Nuclei were stained using Heochst. Immunostaining of LAMP II, Sialidase and Heochst was visualized using the Leica TCS S5P. MG132 treatment increases Sialidase staining resulting in a globular organization as opposed to a reticular formation. (B) Quantification of co-localisation using Mander's Coefficient is illustrated graphically for between five and 14 cells. Treatment of fibroblasts with MG132 leads to an increase in colocalisation as determined by Mander's Coefficient. All images were acquired at 630× magnification. Test of significance was determined using Anova and Bartlett's test for equal variance. (***) represents a significance of $p<0.001$ when compared to the no treatment control. Error bars represent SEM.


A)
LAMPII
NEU1
HEOCHST
Overlay
No Treatment
MG132
Celastrol
MG132 + Celastrol

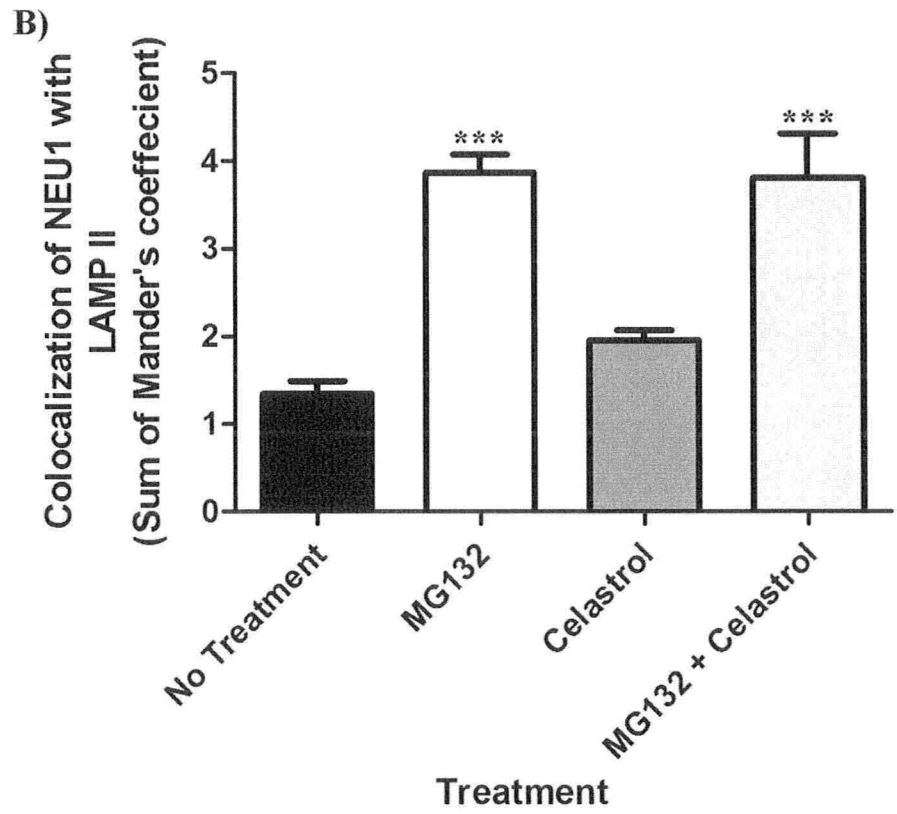
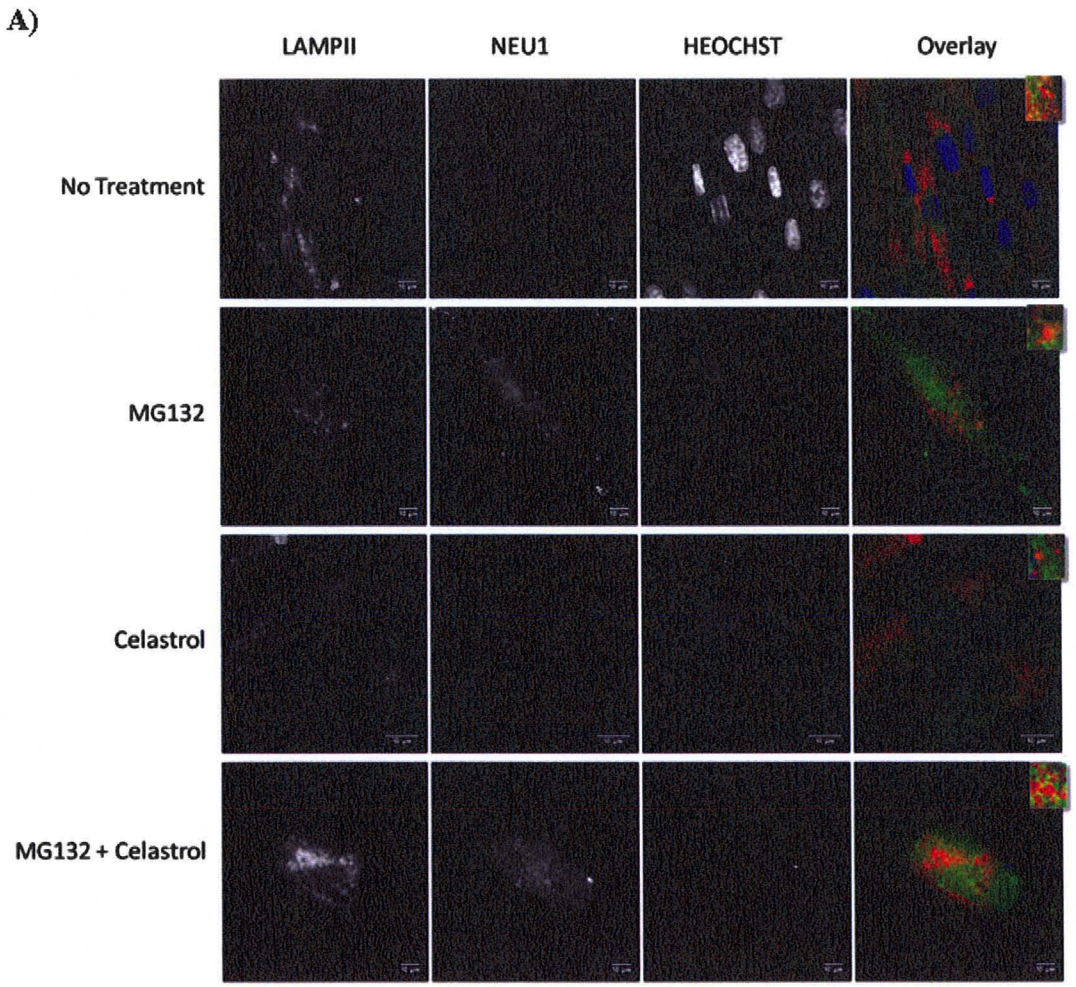


Figure 3.10: **Assessment of the intracellular co-localisation of Sialidase with the lysosomal marker LAMP II in Sialidase-null fibroblasts expressing the mutant R341G Sialidase.** (A) Human Sialidase-null fibroblasts infected with AdSial^{R341G} were treated with MG132, Celastrol or a combination of both MG132 and Celastrol, fixed, permeabolized and immunostained for Sialidase and LAMP II. Nuclei were stained with Heochst. Immunostaining of LAMP II, Sialidase and Heochst was visualized using the Leica TCS S5P. An increase in Sialidase staining occurs in the after treatment with MG132 and staining resembles a punctate pattern with increased organization in cells. Celastrol does not impart any significant change in staining. (B) Quantification of co-localisation using Mander's Coefficient is illustrated graphically and treatment of fibroblasts with MG132 produces an increase in colocalisation however. Celastrol produces limited effect on intracellular localisation. Although intracellular localisation of Sialidase to the LAMP II positive lysosome in fibroblasts treated with both MG132 and Celastrol increases significantly above the no treatment control, this improvement does not surpass that seen after treatment with MG132 alone. All images were acquired at 630× magnification. Test of significance was determined using Anova and Bartlett's test for equal variance. (***) represents a significance of $p<0.001$ when compared to the no treatment control. Error bars represent SEM.


A)
LAMPII
NEU1
HEOCHST
Overlay
No Treatment
MG132
Celastrol
MG132 + Celastrol

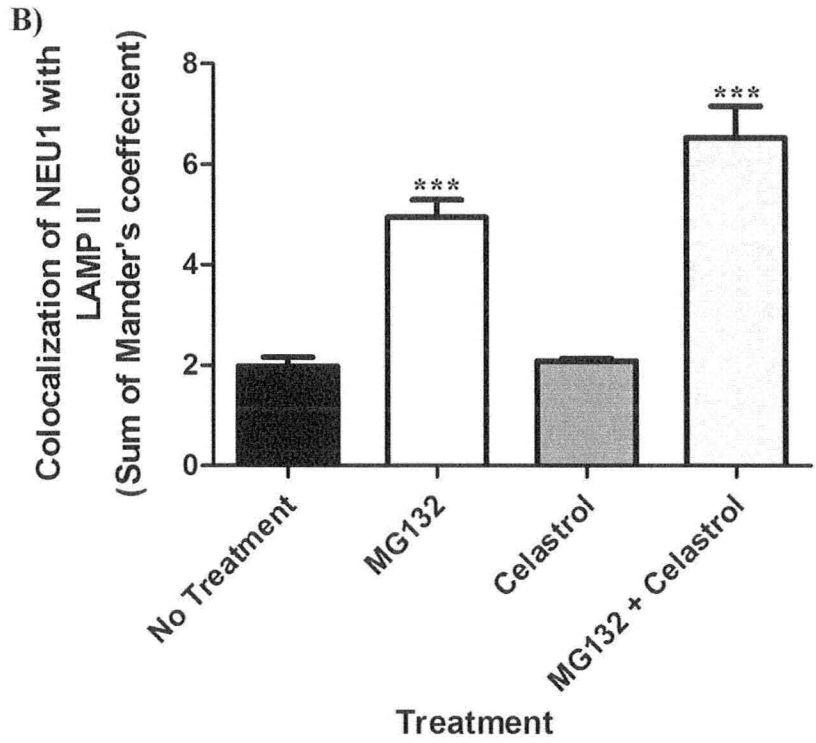
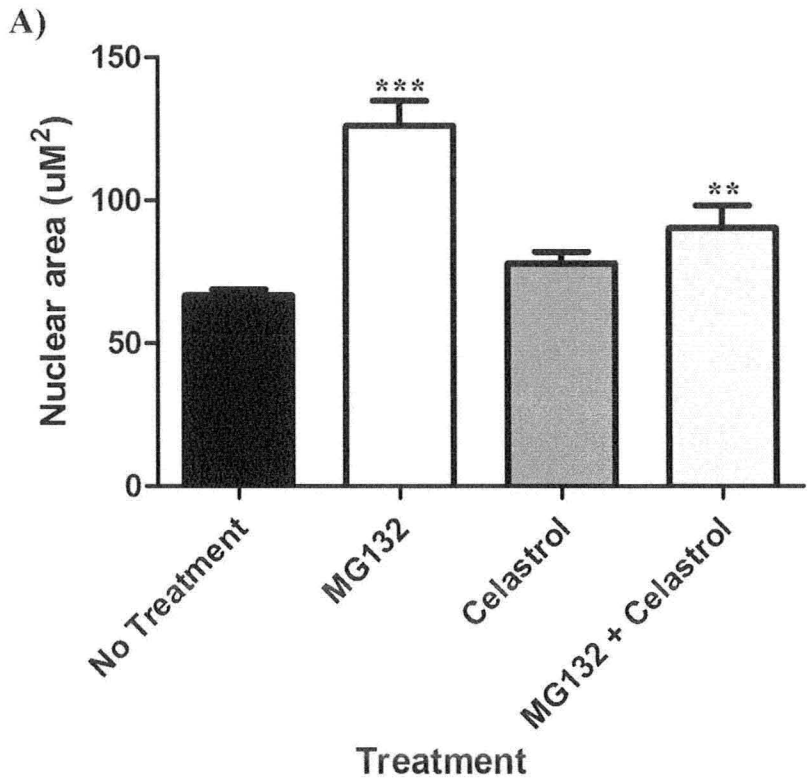
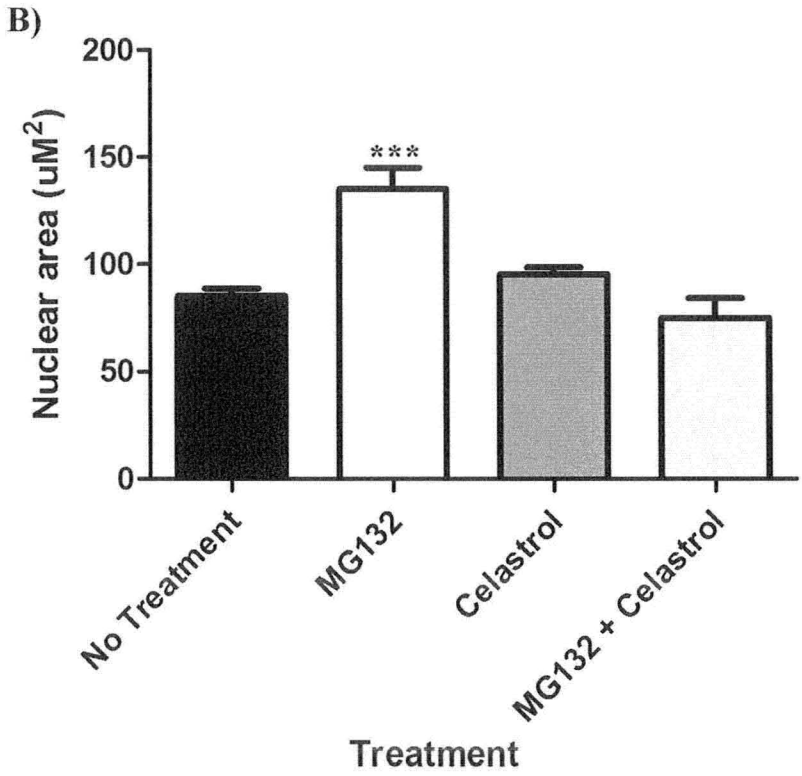


Figure 3.11: **Assessment of the intracellular co-localisation of Sialidase with the lysosomal marker LAMP II in Sialidase-null fibroblasts expressing normal Sialidase.** (A) Human Sialidase-null fibroblasts expressing normal Sialdiase cDNA were treated with MG132, Celastrol or a combination of both MG132 and Celastrol, fixed, permeabolized and immunostained for Sialidase and LAMP II. Nuclei were stained using Heochst. Immunostaining of LAMP II, Sialidase and Heochst were visualized using the Leica TCS S5P. Untreated cells do show punctate formation when expressing normal Sialidase, and we see an increase in staining with MG132 treatment. Celastrol treatment produces a decrease in staining of Sialidase. (B) Quantification of co-localisation using Mander's Coefficient is illustrated graphically. After treatment with MG132, colocalisation of Sialidase with lysosomes increases significantly, while Celastrol does not have a significant effect on targeting of Sialidase. A synergistic effect with treatment of MG132 with Celastrol is evident in cells expressing normal Sialidase as there is a significant improvement in colocalisation of Sialidase with LAMP II in cells treated with MG132 and Celastrol over those treated with MG132 alone ($p<0.05$). All images were acquired at 630× magnification. Test of significance was determined using Anova and Bartlett's test for equal variance. (***) represents a significance of $p<0.001$ when compared to the no treatment control. Error bars represent SEM.


A)
150
100
50
0
Nuclear area (uM^2)
***
**
No Treatment
MG132
Celastrol
MG132 + Celastrol
Treatment


B)
200
150
100
50
0
Nuclear area (uM^2)
***
No Treatment
MG132
Celastrol
MG132 + Celastrol
Treatment

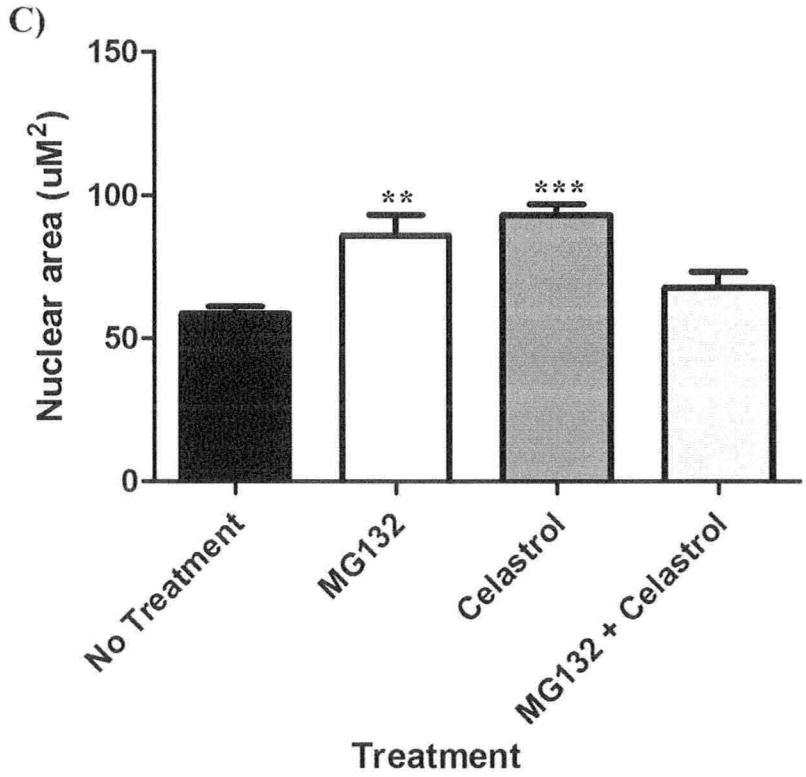


Figure 3.12: **Variations of nuclear area in human Sialidase-null fibroblasts expressing normal or mutant Neu1 cDNA after treatment with MG132, Celastrol or both.** Human Sialidosis cells were infected with AdSial^{+}, AdSial^{R225P}, or AdSial^{R341G} and treated for 72 hours with 0.6μM MG132, 0.6μM Celastrol or 0.6μM MG132 and 0.6μM Celastrol in combination. Cells were fixed and stained with Heochst to visualize nuclei. Measurement of nuclear area was performed on a Zeiss inverted fluorescent microscope. Cell nuclei were measured in cells infected with AdSial^{+} (A), AdSial^{R225P} (B) or AdSial^{R341G} (C). Nuclei are measured in μm^2 using between seven and 84 cells from at least two fields of view. In the normal Neu1 cDNA expressing cells, the nucleus increases in size after treatment with MG132 ;treatment with the combination of both MG132 and Celastrol has a more significant effect on cell size than MG132 alone. The cells infected with AdSial^{R225P} show a cell size increase only in cells treated with MG132 alone whereas the fibroblasts infected with AdSial^{R341G} show increases in size after treatment with MG132 and Celastrol independently but not in combination. Overall, MG132 treatment increase the size of nuclei significantly. Test of significance was determined using one way anova followed by tukey's multiple comparison test. (**) represents $p<0.01$ and (***) represents $p<0.001$ in comparison to the no treatment control. Error bars represent SEM.

3.7 Effects of disease causing mutations on ganglioside accumulation in human fibroblasts loaded with a ganglioside mixture and infected with adenovirus carrying recombinant Sialidase cDNA.

Previously, Pattison et al. described novel mutations of Sialidase and identified the molecular pathology of both the p.R225P and p.R341G substitutions[86]. Further characterization of the abnormalities in ganglioside catabolism as a result of these mutations in vitro could connect Sialidase defects with the sialylglycoconjugates that accumulate in patient's tissues. To identify changes in ganglioside accumulation in human fibroblasts expressing both normal and mutant alleles of Sialidase, Sialidase-null fibroblasts loaded with 200μg of gangliosides and infected with AdSial^{+} at an MOI of 100 or 200 and AdSial^{R341G} at an MOI of 10 or 100 were utilized. Gangliosides and glycolipids were isolated and separated on a silica TLC. In comparison to the Resorcinol positive bands observed in the no virus control, the AdSial^{+} (MOI 200) shows a decrease in all glycosphingolipds stained on the TLC plate (Fig. 3.13). The AdSial^{+} (MOI 100) does not show decreases, however the accumulation does increase with the addition of the AdSial^{+} (MOI 100). Upon examination of the ganglioside banding pattern of cells infected with AdSial^{R341G} there is an increase in complex glycolipids migrating directly below GM1 when compared to uninfected control. The glycolipids seen directly above GM2 are less complex and possess less negative charge; these gangliosides show decreased intensity after Resorcinol staining. When comparing cells infected with AdSial^{+} (MOI 100) to those infected with the AdSial^{R341G} (MOI 10), an increase in the intensity of low and high complexity gangliosides is observed in cells expressing the mutant Sialidase allele. Of note, immunoblotting for Sialidase expression revealed an MOI dependent increase in Sialidase expression in cells infected with AdSial^{+}; however, in the cells infected with the AdSial^{R341G} (MOI 100) there was a limited Sialidase expression. Cells

infected with AdSial^{R341G} (MOI 10) show a high level of Sialidase expression, equivalent to the expression of Sialidase in cells infected with AdSial^{+} (MOI 200) (Data not shown).

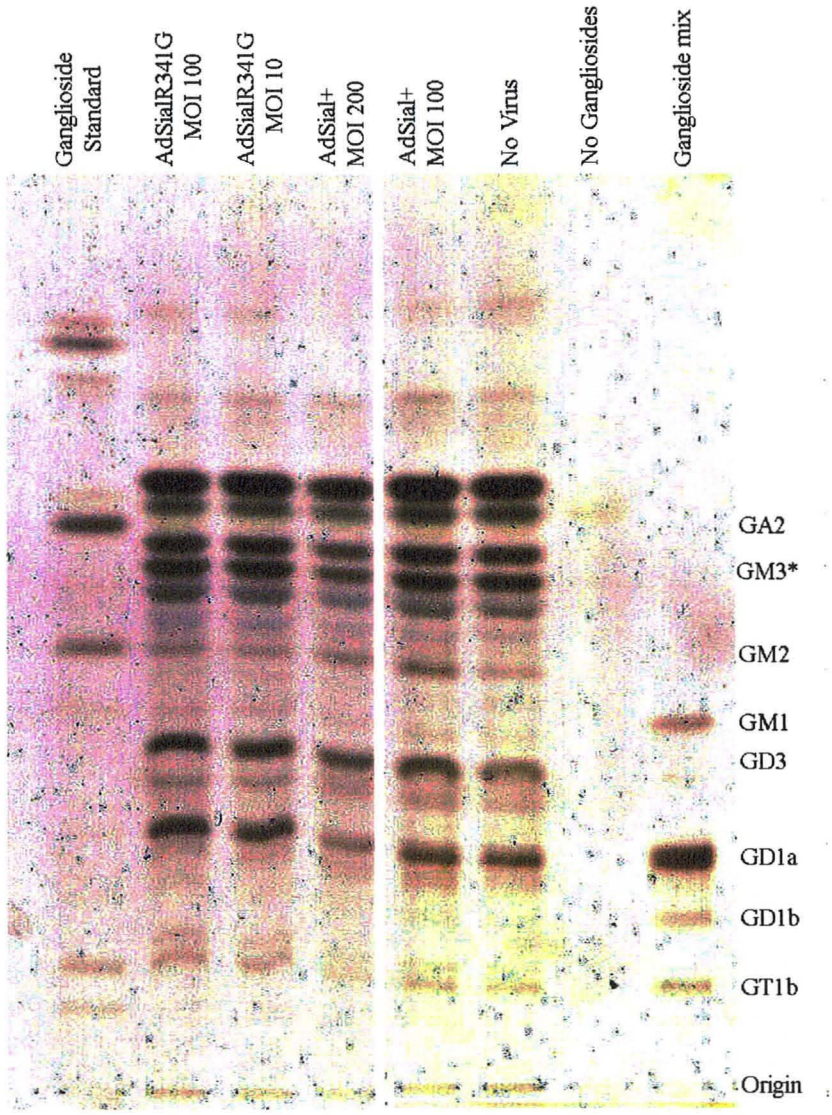

Ganglioside Standard
AdSialR341G MOI 100
AdSialR341G MOI 10
AdSial+ MOI 200
AdSial+ MOI 100
No Virus
No Gangliosides
Ganglioside mix
GA2
GM3*
GM2
GM1
GD3
GD1a
GD1b
GT1b
Origin

Figure 3.13: **TLC analysis of gangliosides and glycolipids isolated from human Sialidase-null fibroblasts pre- loaded with gangliosides and expressing normal and mutant Sialidase.** Human Sialidase-null fibroblasts were grown to confluency and loaded with 200μg of a bovine brain mixture of gangliosides in Opti-MEM for 5 days. After infection with adenoviruses expressing recombinant normal or c.1021C>G (p.R341G) mutant Neu1 cDNA at varying MOI for 3 days, cells were harvested and glycoconjugates were isolated using Chloroform:Methanol extraction along with saponification and size exclusion filtering. Purified lysates were blotted onto a silica TLC plate and sprayed with Resorcinol/H_2SO_4 for visualization of glycolipids. Image was captured using a UV camera and adjusted for print. GM3* represents the putative location of GM3 on the TLC. Clearance of glycolipids is evident in the lane blotted with gangliosides isolated from cells infected with AdSial$^+$ at an MOI 200. Although not a clear change in catabolism of gangliosides is evident in cells expressing the c.1021C>G (p.R341G) cDNA there are small changes in GM2 staining as well as some increases in higher complexity glycoconjugates and small decreases in lower complexity-positively charged glycolipids.

3.8 Effect of treatment with MG132, Celastrol or both on ganglioside catabolism in human Sialidase-null fibroblasts pre-loaded with ganglioside mix and expressing normal Neu1 cDNA

As a natural ligand for Sialidase in human somatic and neuronal cells, ganglioside accumulation is likely a determinant for disease severity. To test the effects of treatment with MG132 or Celastrol on catabolism of gangliosides, human Sialidase-null fibroblasts were loaded with a mixture of gangliosides and co-infected with AdSial^{+} at an MOI of 100 and an adenovirus expressing Protective Protein Cathepsin A (MOI 10). Previously, we observed a small change in catabolism of gangliosides with the addition of transiently expressed normal Sialidase cDNA (Fig. 3.13). We hypothesized that the unexpectedly minor effect of normal Sialidase cDNA on catabolism of gangliosides could be due to a limited amount of Protective Protein Cathepsin A available for the excess of Sialidase in infected cells. Protective Protein Cathepsin A (PPCA) and β-galactosidase form a multi-protein complex with, and is required for the activation of Sialidase in cells[122]. To counteract the potentially limiting amount of PPCA, cells were co-infected with an adenovirus carrying a bicistronic vector expressing Protective Protein Cathepsin A (PPCA) at an MOI of 10. This adenovirus carries the PPCA and Sialidase cDNA separated by the encephalomyocarditis virus internal ribosomal entry-site (EMCV-IRES) allowing for translation of both the PPCA followed by Sialidase (CIRES)[85] (Fig. 3.14). After co-infection with both AdSial^{+} and AdSial$^{\text{CIRES}}$, fibroblasts received treatment with 0.6μM MG132, 0.6μM Celastrol or both 0.6μM MG132 and 0.6μM Celastrol in DMEM for 72 hours. After treatment cells were harvested in water and gangliosides and glycolipids were run on a silica TLC plate. Ganglioside staining in the AdSial^{+}/AdSial$^{\text{CIRES}}$ no treatment lane is reduced for most species in comparison to the no virus control. Of note, there is the generation of a band above the Tri-sialylated GT1b

clearly visible in the cells expressing normal recombinant Sialidase. Also a decrease in intensity is evident in the GM1 band as well as species surrounding GM1. GM2 is almost entirely depleted in cells infected with AdSial^{+}/AdSial$^{\text{CIRES}}$ as well as the less complex species above GM2. Finally we see a clear depletion of bands surrounding the Asialo-ganglioside GA2 (Fig. 3.15). Catabolism changes seen in the cells co-infected with AdSial^{+} and AdSial$^{\text{CIRES}}$ differs from the ganglioside band pattern identified after Sialidase-null fibroblasts are infected with AdSial^{+} alone (Fig. 3.13).

Treatment of cells expressing the transient normal Sialidase and Cathepsin A cDNA with MG132 and Celastrol further changes the ganglioside catabolism of loaded fibroblasts with null Sialidase. The band corresponding to GM1 is dramatically decreased in comparison to the no treatment control when cells are treated with MG132, on the other hand the species corresponding to GM2 seems to be increased above the virus infected no treatment samples as well as the no virus no treatment control (Fig. 3.15). An unidentified ganglioside species directly above GM2 is almost completely depleted while the doublet immediately above this species is visibly decreased after treatment with MG132.

Celastrol has already been shown to have an effect on highly complex gangliosides in the Trisialo- and Disialo- series of gangliosides when used on endogenous levels of Sialidase in a Sialidosis patient cell line (Fig. 3.4). Again, Celastrol depletes the GT1b band in comparison to both the no virus control and AdSial^{+}/AdSial$^{\text{CIRES}}$ after no treatment. However, Celastrol effects no significant change in the banding pattern beyond the high complexity species. The synergistic effect of treatment with both MG132 and Celastrol is clear in examining the changed banding pattern in comparison to both the No Virus control and the No treatment lanes. Firstly, there is a definitive decrease in banding intensity, even in comparison to the No treatment lane across most of the species resolved on the TLC. The effect of Celastrol on the higher complexity species is mirrored in the combination treatment as well as the decreases seen in cells treated with MG132 alone including changes in GM1 and the less complex gangliosides. Beyond mirroring the effects of each of the independent drug

treatments, the combination of MG132 and Celastrol has also decreased the less complex species that migrate faster on the TLC than GA2 pointing to the overall catabolism of the complete sialylated and asialo- ganglioside series.

Figure 3.14: **Schematic representation of the bicistronic vector expressing both Cathepsin A and Sialidase under the ECMV-IRES control.** Modified from Pattison, 2007[85]

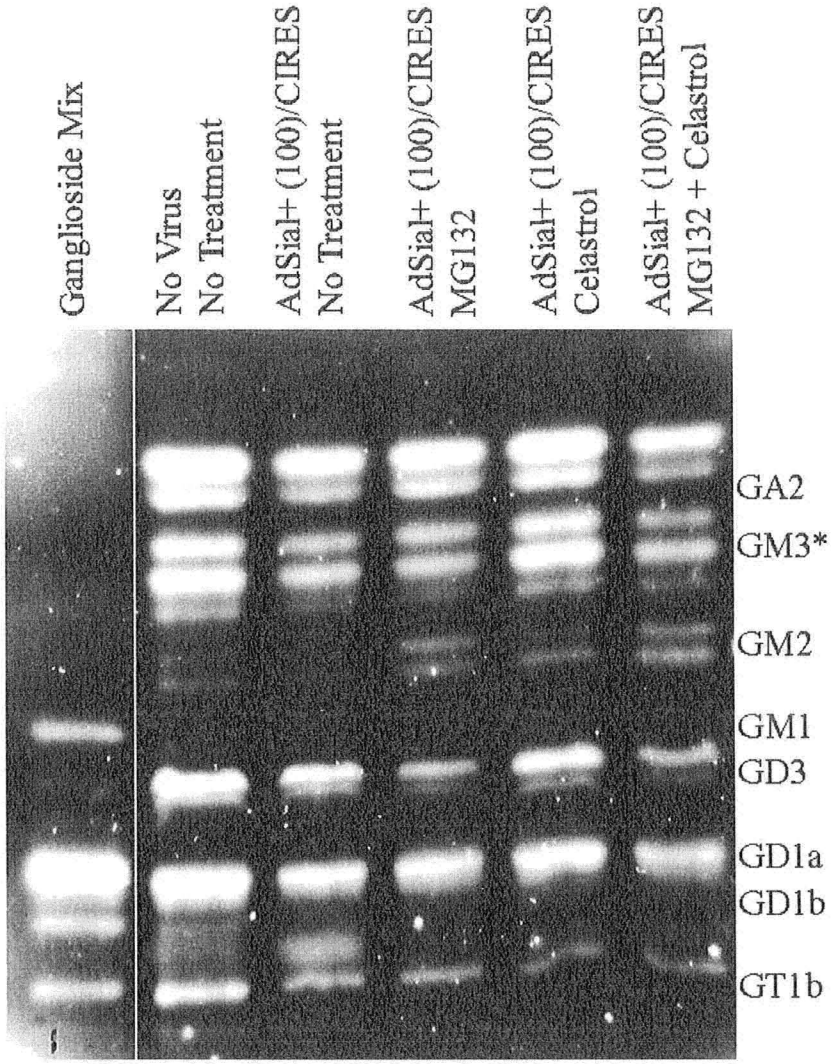

Ganglioside Mix
No Virus
No Treatment
AdSial+ (100)/CIRES
No Treatment
AdSial+ (100)/CIRES
MG132
AdSial+ (100)/CIRES
Celastrol
AdSial+ (100)/CIRES
MG132 + Celastrol
GA2
GM3*
GM2
GM1
GD3
GD1a
GD1b
GT1b

Figure 3.15: **Ganglioside levels in human Sialidase-null fibroblasts expressing normal Sialidase cDNA along with human Protective Protein Cathepsin A cDNA after treatment with MG132, Celastrol and both MG132 and Celastrol in combination.** Human Sialidase-null cells were grown to confluencey and loaded with 200μg of a bovine brain mixture of gangliosides followed by co-infection with AdSial^{+} and adenovirus carrying the CIRES vector expressing human Protective Protein Cathepsin A. After co-infection cells were incubated with MG132, Celastrol, or both MG132 and Celastrol for 72 hours. After harvesting lysates in water glycolipids and gangliosides were purified using Chloroform:Mathanol, saponification and size exclusion filtration. Purified sialylglycoconjugates were blotted on a silica TLC plate and bands were stained with Resorcinol/H_2SO_4. Lane loading was equalized using protein levels determined by Lowry assay. GM3* represents the putative location of GM3 on the TLC. A clear reduction is evident with the transient expression of both normal Sialidase and Cathepsin A cDNA. MG132 does reduce GD3 and the species less complex than GM2 migrating faster up the plate. Celastrol has a positive effect in reducing the higher complexity gangliosides including GT1b. The synergistic effect of treatment with MG132 and Celastrol can be seen in reduction of bands affected by individual treatments as well as a general decrease in band intensity in comparison to both the uninfected and no treatment lanes.

Chapter 4

Discussion

Sialidosis is a rare and highly unpredictable disease affecting muscles, organs and inducing neurodegeneration in inflicted patients[16,67]. The heterogeneity of the disease phenotype is inextricably linked to the extensive variation seen amongst mutations of Sialidase, whose malfunction leads to the accumulation of sialylglycoconjugates and oligosaccharides which are biomarkers of this devastating disease[16,67,70,105]. Investigations into treatment options for Sialidosis have been hindered by the variation in both genotype and phenotype with current treatment being limited to symptom management.

Cloning of the gene encoding the Sialidase enzyme followed by its characterization allowed for major advances in understanding the pathology of Sialidosis and how mutations in the NEU1 gene can lead to enzyme malfunction and misfolding[8,9,16,47,69,105]. Using the highly homologous NEU2 (cytosolic Sialidase) as a reference, general studies were able to interpret specific mutations according to their proximity to conserved regions, potential active sites or motifs involved in the formation of the complex with Cathepsin A and β-galactosidase[12,69,70,73]. Through this homology analysis, mutations of Sialidase were partitioned into three classes: those that cause protein truncation or fail to produce a protein at all, those that result in a catalytically inactive protein, and finally, misfolded proteins which are retained in the ER and eventually degraded by the proteasome[79]. Through the classification of Sialidase mutations, we are able to establish a connection between disease phenotype, accumulation products, and genotype.

Diseases caused by misfolding proteins include aggregation disorders like: Alzheimer's, Parkinson's and Huntington disease as well as the lysosomal storage disorders[38,41]. These misfolded proteins are exciting targets for therapy development, in that a protein can potentially retain catalytic activity despite lost functionality in the misfolded state. Through the use of chaperones, proteosomal regulators and small pharmacologicals, researchers have been targeting these misfolded proteins to develop new therapies[5,25,81,100,128].

Proteosomal regulation is one of the possible treatment options currently under investigation for disorders of protein misfolding. In order for the cells to control misfolded

proteins, the proteasome works along with the Endoplasmic Reticulum Associated Degradation (ERAD) and the Unfolded Protein Response (UPR) to maintain cell function during times of cellular stress[88,94]. The 26s proteasome is comprised of two subunits: the 19s regulatory unit and 20s proteosomal core. Proteosomal regulation using pharmacological agents allows for ubiquitinated proteins to accumulate in the absence of the proteasome. Protein accumulation leads to the upregulation of Heat Shock Proteins (HSPs) and initiation of ERAD[15]. Upon treatment with proteosomal regulators such as MG132, or with Celastrol treatment, there is a marked upregulation of HSP 70 and 90 as well as an initiation of Heat Shock Factor 1 (HSF1). HSF1 regulates the transcription of the cell's natural chaperones which assist in folding proteins accumulating in the ER and Cytosol[75,126].

We explored the potential of exploiting MG132 and Celastrol for their ability to initiate the UPR and prevent misfolded protein degradation, as well as upregulatng the cells natural chaperones. Mu et.al.(2008) determined that MG132 and Celastrol have an impact on the presence of chaperones and folding mechanisms that accompany ERAD[81]. Using previously identified mutations of Sialidase[86], as well as human primary Sialidosis cells, we assessed the potential of proteosomal inhibition to recover the metabolic phenotype of each Sialidase mutation as well as postively impact the endogenous enzyme in vitro.

Initially, we examined the effect of protein regulation on Sialidase enzyme activity in Sialidosis cells. The patient cell line used expressess the c.3G>A (p.M1?) and c.1021C>G (p.R341G) mutant cDNA[86]. The p.M1? substitution results in a premature stop codon and the protein is thus truncated and inactive. The p.R341G mutant allele results in a properly targeted protein, however, the lack of the positively charged Argenine (R) is believed to abolish the protein's ability to bind to the negative sialic acid on target ligands and is therefore, catalytically inactive[86]. The Sialidosis cell line displays a characteristically low enzyme activity in untreated fibroblasts (approximately 13%) in comparison to the reference MCH64 normal cells[67]. Specific enzyme activity, measured using the artificial substrate MuNANA, is recovered beyond that of the normal MCH64 cells after treatment

with both MG132 and Celastrol (Fig. 3.3). Given that the p.M1? is truncated, we can assume all observed treatment effects are due to changes in the functionality of the R341G mutant Sialidase. MG132's ability to shut the proteasome down, effectively allows for the mutant allele gene product to remain in excess. Since Sialidase with the R341G substitution does transport effectively to the lysosome[86], we predict that the protein enters the low pH environment within the lysosome and is available for folding and binding by the sialyl-glycoconjugates. Any increases in enzyme activity or localization of Sialidase is imparted by sheer volume of protein available in the absence of the proteasome. With the increase in available activity, we need to resolve the issue surrounding the absence of a postively charged amino acid within the putative active site. After examination of the conserved amino acids surrounding the substituted residues, we see a highly conserved histidine at position 337[8,86]. As histidine is known to become protonated in environments of low pH, we postulate that the H337 may provide the positive charge required at the putative active site, absent after the substitution of argenine for proline[69]. The availability of a positively charged residue proximal to the mutated active site could be responsible for the increased activity observed in Sialidosis cells.

After observing the positive change in enzyme activity after treatment, we proceeded to examine if the treatment of Sialidosis cells with MG132 and Celastrol will also increase targeting of the endogenous enzyme to the lysosome. The targeting mechanism of Sialidase to the lysosome is under continued investigation; with potential targeting pathways using the Mannose 6-phosphate receptor or the adaptor protein AP3[71]. Sialidase has been tracked from the trans-golgi network to lysosomes through the endosomal/lysosomal pathway using a targeting motif (YGTL) identified at the C-terminus. In the absence of targeting motif, Sialidase is retained in the plasma membrane[71]. As misfolded proteins accumulate, lysosomal targeting is hindered and proteins are retained in the plasma membrane or trapped in the ER or Golgi producing a reticular staining seen in immunohistochemistry[8,70,86]. Without high transient expression from an exogenous source (i.e. adenoviral infection) of mutant

Sialidase it is difficult to observe enzyme levels in Sialidosis patients. Bonten et.al. (1996) determined that the level of enzyme staining in Sialidase patients falls below the level of detection[8]. A similar absence of staining is seen in our experiments involving Sialidosis cell lines. With the addition of MG132 however, Sialidase staining increases and although there is reticular staining evident, there is an increase in perinuclear punctate visible, as was seen previously[8]. Increases in reticular staining of Sialidase can be associated with with an abundance of enzyme held in the ER as the cell attempts to processes the excess Sialidase with only endogenous levels of PPCA[10,70,128]. Using Mander's Coefficient, which identifies the overlap of one fluorescence channel (red), representing Neu1, with another (green), representing LAMP II, we determine that the localisation of Sialidase with the lysosome is increased after treatment with MG132. The additive effect on enzyme activity we see in cells treated with both MG132 and Celastrol is not echoed in our examination of colocalisation of Sialidase with the lysosomal marker, LAMP II.

The role of Sialidase in cells is to hydrolyse sialic acid primarily linked via an α2-3 configuration from oligosaccharides, glycolipids and glycoproteins. The accumulation of gangliosides in Sialidosis patients is marred in controversy, with conflicting reports of gangliosides as accumulated substrates, and continued efforts to clarify the role of Sialidase in ganglioside catabolism[48,65,120]. There is evidence that Sialidase plays a role in the catabolism of gangliosides, and that ganglioside accumulation is observed in the absence of active Sialidase[18,47,120,127]. Having established the effect of proteosomal regulation on the enzyme activity we wish to identify if the efficiency of Sialidase is improved by examining the relative levels of putative natural substrate gangliosides. In particular we focus on GM3, GD1a, and GD3, who's accumulation have been observed in Sialidosis patients [65,102,127,129]. Previously, it has been postulated that the response of enzyme activity on the artificial substrate MuNANA can be less specific in comparison to the enzyme effect on the natural ligands *in vitro*[49]. To examine changes in enzyme activity toward gangliosides, we pre-loaded Sialidosis fibroblasts with a bovine brain mixture of gangliosides. Fibroblasts

do not generate endogenous gangliosides, therefore, pre-loading of fibroblasts is a common practice to study glycolipid patterns in vitro. Fibroblasts do have the ability to take-up and break-down gangliosides made available in media[104] as shown previously by TLC analysis in Tay-Sachs disease cells[20,121]. Using loaded fibroblasts we hope to elucidate the mechanism of ganglioside accumulation in Sialidosis cells expressing endogenous levels of Sialidase.

After isolation, purification and blotting on silica TLC plates, we were able to identify an array of gangliosides and glycolipids in the Sialidosis cells. The GD3, GM2 and GA2 gangliosides are clearly evident as well as the more complex negatively charged gangliosides GT1b and the gangliosides of the Di-sialo series. Of note, there is an accumulation of lipids amongst the species between GM2 and GA2. GM3 has been observed as a doublet just above GM2 on a TLC separated using Chloroform: Methanol[20,72]. We can also claim that GM3 would be seen between the GM2 and GA2 bands, because when we examine ganglioside structure we know that GM3 ((α2 - 3)-NeuAcGal(β1 - 4) - Glc - Cer) is a smaller glycolipid than GM2 because of the loss of Galactose, and is more negatively charged and larger than GA2 because of an extra sialic acid side chain[58]. Understanding the biochemical reaction of the TLC we assume that smaller glycolipids will migrate faster and the negative charge of the sialic acid will slow migration up the plate. Using known ganglioside mixtures we identified the accumulation of gangliosides in Sialidosis cells, and determined the effect that treatment has on that accumulation pattern.

Initially, we note that treatment with MG132 results in a decrease in the GD3 band, as well as the GD1a band. The GD3 band, seen as a doublet in the untreated Sialidosis cells, is reduced to one single size band with a decreased intensity. GD3 is expected to be reduced to GM3 by Sialidase[99], which is then further catabolised in the presence of MG132. This reduction of GD3 is imparative, as GD3 has been implicated in the apoptotic cascade, and preventing its degredation could reduce apoptosis in deseased cells[40]. GM2 also decreases, leading us to presume that the catabolic pathway is functioning in these cells. Evidence has been presented that Sialidase degrades GM2 into GA2 in mice, but

there is no evidence showing the same pathway existing in humans as of yet[48]. However, with MG132 we see a decrease in the level of GM2, possibly due to Sialidase, although we don't see a subsequent increase in GA2. Treatment with Celastrol leads to an absence of the more complex gangliosides including GT1b and a decrease in GD1b intensity (Fig. 3.4). These gangliosides are typically degraded by a plasma membrane associated Sialidase[61]. This plasma membrane associated Sialidase activity could be attributed to NEU3 (plasma membrane sialidase) or Sialidase given the identification of a plasma membrane associated pool of lysosomal Sialidase. Celastrol improves the ability of Sialidosis cells to catabolise GT1b and GD1b. After examination of ganglioside accumulation patterns of Sialidosis cells treated with either MG132 or Celastrol, we see that each drug imparts unique effects, and that these effects are simultaneously observed when cells are treated with both drugs in combination.

Celastrol, a natural anti-inflammatory agent, imparts a variety of effects in cellular pathways. It has been shown that Celastrol can act as a proteosomal regulator through interaction with the thiol group of (C) cysteines within the proteasome rendering it inactive [81,97]. However, because Celastrol is producing a unique effect on Sialidase in comparison to the highly specific proteosomal regulator MG132, we can assume the effects of Celastrol on Sialidase is not only due to proteosomal inhibition. Celastrol also prevents aggregation of proteins in Huntington animal models as well as reducing amyloid plaque formation in the brains of Alzheimer mouse models and cell lines[84,128]. The action of Celastrol in both of these diseases is not well understood, however it is believed that Celastrol's inhibition of NFκB formation prevents aggregation. Heat Shock Proteins are also increased in the presence of Celastrol, which induces HSF1[132] and can prevent aggregation. We also know that Celastrol's immunosuppressive activities can be attributed to a reduction of TNFα as treatment with Celastrol has previously shown this[24]. Sialidase activity has been inextricably linked to the production of TNFα and activation of CD44 in stimulatedimmune cells. As a decrease in TNFα reduces activated Sialidase, we can attribute Celastrol's effects on

enzyme activity to its ability to control Sialidase through the immune cytokines[37,53]. Also, Celastrol at lower concentrations (below 1μM) requires a second stressor (e.g. heat shock) to initiate the HSP up-regulation, therefore, protein accumulation in Sialidosis cells in the presence of MG132 could initiate a Celastrol mediated HSP increase[132]. On its own Celastrol fails to affect enzyme activity and expression of Sialidase, however, in the presence of a proteosomal regulator like MG132, the increase in chaperones available through the activation of HSF1 could be beneficial. This HSP response, along with the prevention of aggregations and the excess of Sialidase protein available can explain the additive effect we have seen thus far.

A better understanding of how treatment affects the phenotype/genotype association in Sialidosis can determine if treatments must be mutant specific or can be a more global option. The effect of proteosomal regulation on specific alleles of Sialidase can be analyzed using adenovirus delivered recombinant mutant Sialidase identified in Sialidosis patients[86]. Two mutations of interest; the p.R225P (c.1021C>G) substitution and p.R341G (c.674G>C) substitution were characterized in relation to their misfolding, localisation and enzyme activity[86]. After comparison with homologous human forms of Sialidase and species conserved residues of the Sialidase protein it was postulated that the residue R225 is involved in the stabilization of Sialidase after folding in the ER[86]. Substitution of the proline (P) for the argenine (R) residue leads to misfolding and reduced catalytic activity. The argenine (R) residue at position 341 was determined to be a key conserved amino acid for proper attachment and stabilisation of the sialic acid residue of the substrate. The introduction of a glycine (G) in place of the argenine ensures a loss of a positively charged amino acid, and a loss of the ability to bind to the negatively charged sialic acid[79,86]. Sialidase activity assays of the mutant alleles expressed in Sialidase-null cells revealed that the activity of each mutant allele is significantly increased above that of no treatment controls when treated with MG132 (Fig. 3.5). Again we observe an additive increase in Sialidase enzyme activity in cells treated with both MG132 and Celastrol, indicative of apositive effect of

both the excess protein and available HSPs in cells infected with AdSial^{R225P}.

Proper folding of Sialidase occurs in the endoplasmic reticulum before forming a complex with PPCA and β-galactosidase in the Golgi[10]. Should misfolding of Sialidase occur, the formation of the complex needed for optimal catalytic Sialidase activity will be negatively affected. In cells treated with MG132, we revealed increased expression of a small isoform of the ER chaperone, Grp78 (Figure 3.6A-3.8A). The 72 kDa isoform, which has been identified as a degradation product of the 78 kDa size Grp78, was observed in cells with increased ERAD and UPR[92]. The increase in ER chaperone (with MG132) and cytosolic chaperones (with Celastrol)[132] would lead to the additive effect we see in cells expressing the R225P mutant allele of Sialidase. Therefore, Grp78 may lead to refolding which could lead to appropriate complex formation and eventual targeting to the lysosome. Cells expressing the R341G mutant allele after treatment with MG132, show a significant increase in enzyme activity, equal to that of cells expressing the normal Sialidase allele. MG132 treatment was shown again to not only increase expression of Sialidase but to also increase the expression of the 72kDa species of Grp78 (Fig. 3.7). The availability of excess mutant Sialidase in the absence of the proteasome, combined with the ability of Grp78 to initiate folding, produces the beneficial results we observe. We do not see the same additive effect observed in cells expressing R225P mutant Sialidase; this is most likely due to the fact that the R341G mutation is not strictly a misfolding mutation as much as it is a lack of active site binding by the ligand[70,86]. The chaperone increase we postulate to be Celastrol's key benefit in Sialidosis cells would potentially have a less dramatic effect on the R341G mutant Sialidase.

The expression of Sialidase further elucidates how MG132 and Celastrol effect the allele specific mutations of Sialidase. To examine the expression of Sialidase in the cells expressing both mutant and normal Sialidase cDNA, we blot membranes with an anti-carboxyl Sialidase antibody[47] and anti-RGS His amino terminal antibody[48]. In cells expressing both mutant and normal Sialidase there is an increase in Sialidase expression when blotting with

both the C- and N-terminal antibodies (Fig. 3.6-3.8). This increase leads us to believe that without treatment Sialidase is being degraded by the proteasome. This degredation could be caused by the endogenous PPCA limiting the stability of the excess of endogenously provided Sialidase protein. There is also a C-terminal positive, 15 kDa protein seen in cells expressing both mutant alleles treated with MG132. We know that Sialidase contains an internalisation signal at the C-terminus, which binds to the adaptor protein complexes (AP) in clathrin coated pits[71]. In the absence of this internalisation motif Sialidase is retained at the plasma membrane and not targeted to the lysosome. There are presently two schools of thought regarding how Sialidase interacts with the plasma membrane: either as a protein anchored with a transmembrane region[43,112] or a globular protein attached to the membrane exposing both the C- and N-terminus to either the extracellular/intracellular space. The presence of the 15 kDa protein could be the cleavage product of an endoproteinase which cleaves Sialidase to release it from the plasma membrane, potentially activating it. As a anti-COOH Sialidase antibody positive species only present in cells treated with MG132, we assume this product is normally degraded by the proteasome similar to the proteolytic processing of Presenilin 2 and p105, the NFκB precursor[56,90]. Figure 4.1 shows the two potential cleavage scenarios.

Celastrol treatment in cells expressing either mutant Sialidase, or normal Sialidase alleles all show a clear decrease in expression levels after probing for both the anti-COOH and anti-NH2 antibodies. This expression decrease can be attributed to Celastrol's effect on TNFα, which is an activator of Sialidase activity at the cell surface[37]. This decrease was confirmed in cells expressing the normal Sialidase allele using immunocytochemistry (Fig. 3.11), wherein we see a loss of Sialidase expression after treatment with Celastrol.

After establishing expression patterns after treatment, we then elucidated the effects that treatment has on N-glycosylation which is linked to proper folding and enzyme activity. There are 3 putative N-glycosylation sites found on Sialidase, resulting in species at 45,

47, and 49 kDa[8,86,128]. Treatment with N-glycosidase F, which cleaves N-linked oligosaccharides, results in the compression of the heterogeneous species in mutant alleles, however, this is only observed when there are lower levels of enzyme expression as higher expression levels can diminish this effect[86]. In our experiments, after N-glycosidase F treatment, cells maintain the heterogeneity when treated with MG132 for both mutant alleles (Fig. 3.6-3.8); as the expression is heavily increased in these cells we would not expect to see a collapse of all heterogeneous bands. Lysates treated with MG132 and incubated with the N-glycosidase F, did produce Sialidase species with generally lower molecular weights while maintaining heterogeneity seen in previous studies[86,128]. Size reduction could be due to the loss of N-linked sugars without affecting the banding pattern seen from the 3 putative N-glycosylation sites determined to affect folding[86]. We also observe that the pattern of banding and reduction in size is evident in both the anti-COOH antibody stained lysates as well as the anti-NH2 lysates, showing again that the drugs effect the entire enzyme and are not limited to one particular terminus. We do observe that in cells treated with Celastrol, or both MG132 and Celastrol, there is a reduction of the 3 heterogeneous bands to a more compressed single band after incubation with the N-glycosidase F. This can be attributed to the decrease in Sialidase expression after Celastrol treatment. By determining that MG132 and Celastrol do not hinder the 3 glycosylation sites we can assume that drug treatment does not adversely affect natural folding that is potentially occuring with the aid of heat shock proteins and natural chaperones.

Immunolocalisation was employed to establish that Sialidase is being appropriately targeted to the lysosome to work on available natural substrates (Fig. 3.3). We established that endogenously we do improve targeting of Sialidase but it stands to be determined if treatment can be global, working on multiple alleles of Sialidase. In fact, there is a clear increase in available Sialidase with treatments of MG132 independent of the allele expressed in the cells. Both the R225P and R341G mutant Sialidases show an increase in Sialidase staining after treatment with both MG132 and Celastrol or MG132 alone. The globular

appearance of Sialidase in the R225P mutant allele expressing cells could be attributed to multivesicular bodies that have been identified in transporting Sialidase to the plasma membrane and then to the Lysosome[125]. We have also established that the R225P mutant enzyme activity is more positively impacted by the MG132 and Celastrol treatment in combination. Alternatively, we observe using Mander's Coefficient, that the intracellular targeting of Sialidase to LAMP II positive compartments is greatly improved after treatment with the combination of both drugs, but not above that of treatment with MG132 alone. The increase in colocalisation is promising, however, the presence of large globular accumulations of proteins could be a side-effect of protein accumulation. Mander's Coefficient after treatment with MG132 displays a significant ($p<0.001$) threefold increase in colocalisation of both mutant Sialidases to the lysosomal marker LAMP II, confirming that enzyme activity increases can be attributed active enzyme within the lysosome. Celastrol does produce a minor increase in staining of Sialidase but nothing similar what is seen in cells treated with MG132 and does not improve localisation either on its own or produce an additive effect when cells are co-treated with MG132.

Expression of the R341G protein along with MG132 treatment produces increases in enzyme activity using the artificial substrate; this positive effect is further supported with the localisation studies. In fact, we see that with MG132 treatment there is an increase in Sialidase staining and this staining is in a clear punctate perinuclear pattern (Fig. 3.10). Again we see that Celastrol in isolation is not effective although we do not see the decrease in Sialidase that was shown in the expression analysis. Treatment with both Celastrol and MG132 does not impart an additive effect. This gives further credence to the potential of proteosomal regulation as a global target for Sialidosis treatment strategies.

We also observe a significant increase in nuclear size (Fig. 3.12) and a visible increase in cell thickness after treatment with MG132. This morphological change in size can be accredited to the increase in protein accumulation within the cell in the absence of the proteasome. During UPR and ERAD cells have been shown to change in morphology

to more resemble secretory cells. Increases in the size of the ER and other organelles accommodate increased transcription of ER machinery and accumulating proteins[106].

Ganglioside accumulation in Sialidosis is a controversial concept. Although it is known that mouse lysosomal Sialidase does cleave the α2-3 linked sialic acid from GM2 to produce GA2[48], we still look to identify if this occurs in humans. Researchers have shown that lysosomal Sialidase can cleave sialic acid from GD1a to produce GM1[129], as well as controlling GM3 levels in somatic cells[65,102,127]. We examined whether the introduction of normal Sialidase cDNA into a Sialidase-null cell line (WG544) would uncover the potential to positively affect any observed accumulations of gangliosides in fibroblasts of Sialidosis cells. After observing that expression of normal Sialidase produces both a reticular and punctate staining we can postulate that the allele is being targeted to the lysosome as well as to the plasma membrane as has been seen in previous studies[71]. In both the plasma membrane and the lysosome the Lysosomal Sialidase works on exposed α2-3, α2-6 linkages of sialic acid. Reduction of GM3 and GD3 band intensity in cells expressing normal Sialidase point to potential Sialidase modifications of GD3 to the GM3 product, which continues along the catabolic pathway[98]. Reductions in GD1a are of interest, given that GD1a is not a target of Sialidase, there could be an alternative Sialidase functioning in these cells. There is general reduction of gangliosides accumulation in the AdSial^{+} infected cells providing evidence that normal Sialidase may reverse accumulations. The expression of normal Sialidase in pre-loaded cells was anticipated to completely clear gangliosides; however, we observed only reductions in accumulation. The unexpected maintenance of gangliosides present in cells expressing normal Sialidase could be due to the limiting amount of endogenous Protective Protein Cathepsin A (PPCA) required to complex with Sialidase and β-galactosidase to achieve activation of Sialidase[69]. To investigate this we co-infected cells with both the adenovirus expressing normal Sialidase and an adenovirus with a bi-cistronic PPCA vector (Fig. 3.14). After ganglioside isolation, the AdSial^{+}/AdSial$^{\text{CIRES}}$ co-infected cells displayed a more dramatic decrease in certain species of gangliosides, including a clear reduction of

GM2, GD1a and higher-complexity gangliosides GT1b, and GD1b (Fig. 3.15). As these cells have a normal hexosaminidase A which cleaves the terminal sialic acid from GM2 resulting in GM3, we would not assume an accumulation at GM2[60]. The unexpected accumulation could develop because of a blockage at GM3 preventing the cell from pushing the catabolism process through, and the backlog produces accumulations throughout the entire pathway. With the introduction of normal Sialidase and the reduction of GM3, the entire pathway begins to work more effectively and we see a depletion of GM2. The accumulation of gangliosides and subsequent positive changes in catabolism with transient expression of recombinant normal Sialidase, reveals that gangliosides are potential targets for Lysosomal Sialidase.

Using isolated gangliosides from cells infected with both the AdSial^{+} and the AdSial$^{\text{CIRES}}$ (bicistronic Cathepsin A) after treatment with MG132, Celastrol or both, we examined if ganglioside accumulation is reversed. Cells treated with either drug independently or in combination results in a decrease in accumulated products. After infection with both AdSial^{+} and AdSial$^{\text{CIRES}}$ without treatment, there is also a reduction in certain accumulated species of gangliosides (Fig. 3.15) showing PPCA as a limiting factor in this study. Primarily, we note that treatment with MG132 decreases the GD3 species, which we also observed in the treatment of cells expressing endogenous mutant Sialidase. This decrease in intensity is accompanied by a decrease in the putative GM3 bands. The accumulation of GM3 in cells treated with Celastrol alone is similar to that seen in the no virus control. Decreases in the more complex gangliosides can be linked to Celastrol's immune signaling effect, as the presence of these species, including GD3 is implicated in immune activation[28]. The additive effect of MG132 and Celastrol produces a general decrease of all species of gangliosides, and although we do not see the complete loss of GM3 or depletion of GD3 we observed in MG132 treated cells, we do see a decrease in the less complex bands which migrate faster than GM3. The presence of GD3 and GM3 could be indicative of a constant processing of all gangliosides producing all intermediate species and catabolising them appropriately.

Throughout this study we looked to establish characteristics that could link genotype to phenotype of Sialidosis patients and solidify the therapeutic potential of the pharmacological agents MG132 and Celastrol. We clearly identify proteosomal regulation as a potential therapy target for Sialidosis, as we saw an increase in activity, intracellular localisation and substrate reduction in both endogenous cells and cells expressing mutant alleles of Sialidase cDNA. We also confirmed that proteosomal inhibition is a global treatment, imparting positive phenotypic results to both the R225P and R341G mutant alleles of Sialidase. MG132 also initiated pathways involved in protein folding and maintenance, including an increase in Grp78 isoforms. Celastrol, the natural anti-inflammatory drug, depletes Sialidase expression across both normal expressing and mutant alleles of Sialidase. The link between Sialidase and the immune response points to a novel pathway in which Celastrol inhibits immune signaling. Further study into the downstream effects of Celastrol on Sialidase induced cytokine responses using ELISA could solidify this result. We also observed an additive effect with treatment of MG132 and Celastrol which could be attributed to the positive effect of proteosomal inhibition as well as Celastrols upregulation of ERAD folding machinery. Proteosomal regulation is an excellent candidate for the improvement of Sialidase function in Sialidosis patients and FDA approved Bortezomib (VELCADE®), used in the treatment of multiple myeloma patients, shares a number of inhibitory effects and apoptotic rates with MG132[13,27]. The use of proteosomal regulation is a promising target for future treatment options for a variety of Sialidosis patients.

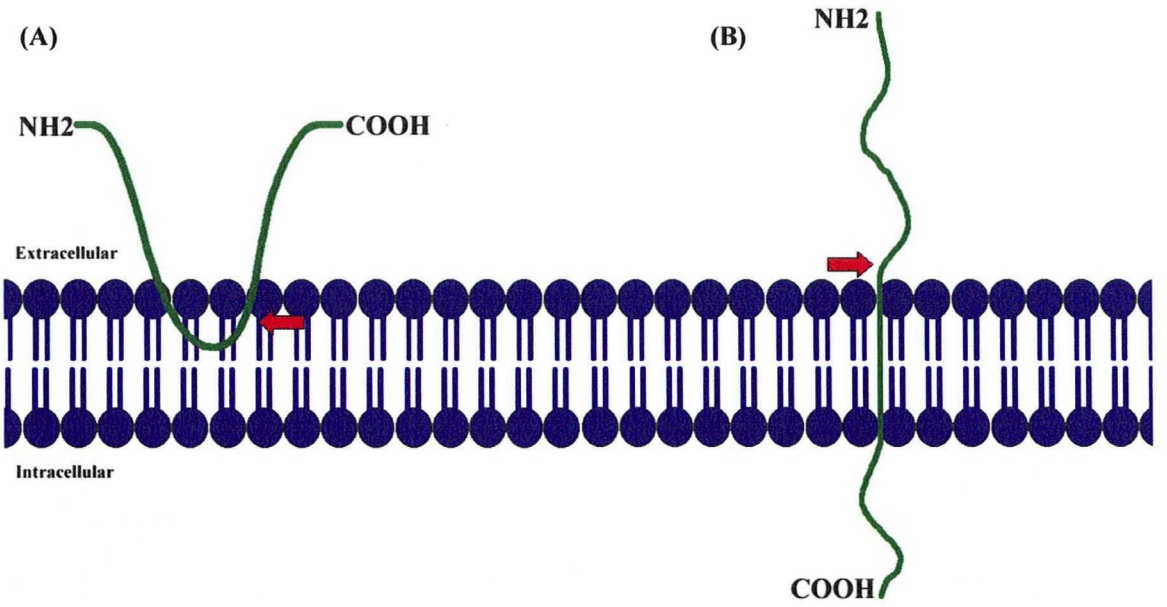


Figure 4.1: **Cleavage possibilities of Sialidase associated with the plasma membrane.** (A) Sialidase associates with the plasma membrane with both the N- and C-termini facing the extracellular space. Cleavage of the protein for activation in signalling cascades effectively produces both an N-terminal species and C-terminal species. (B) Sialidase with a transmembrane domain has the N-terminal section into the extracellular space and the C-terminal end facing the intracellular space (or lysosomal luman as the case may be). At cleavage the N-terminal remains in the plasma membrane and release of the C-terminal end activates the Sialidase and retains the signalling peptide found at the C-terminal end.

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