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The effect of chaperone compounds on glucocerebrosidase stability and activity in fibroblasts from Gaucher Disease patients with different genotypes.

Raquel da Fonseca Gonçalves

Trabalho efectuado sob a orientação de:

Prof. Doutor Gustavo Tiscórnia

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"The whole of science is nothing more than a refinement of everyday thinking"

Mr Albert Einstein

The effect of chaperone compounds on glucocerebrosidase stability and activity in fibroblasts from Gaucher Disease patients with different genotypes.

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Abstract

Gaucher disease is the most common lysosomal storage disorder and may present systemic and neurological symptoms. It is caused by mutations in the *GBA* gene that encodes for glucocerebrosidase (GBA), an enzyme involved in the degradation of sphingolipids in the lysosome of cells. The enzymatic deficiency leads to accumulation of GBA substrate in cell lysosomes. The disease can present three different clinical types: type 1 presents mainly non-neuropathic symptoms in multiple organs, whereas type 2 and type 3 are characterized by neurological complications. The only treatments currently available (enzyme replacement therapy and substrate reduction therapy) treat the visceral symptoms, but not the neuropathic manifestations. An alternative approach uses pharmacological chaperones to improve the affected enzyme activity and restore normal activity. This therapy has the potential to treat both neurological and visceral symptoms.

The main purpose of this thesis is to test the effect of 8 different chaperone compounds on glucocerebrosidase stability and activity. These chaperones were tested in fibroblasts from Gaucher disease patients with 4 different genotypes. The results on this experimental work suggest that the effect of a given chaperone depends on the mutation affecting the GBA protein. The techniques established in this work will eventually be used to test the effect of chaperone compounds on neurons derived from induced pluripotent stem cells (IPSc) of Gaucher Disease patients, as potential therapeutic agents.

Key words: Gaucher Disease; Glucocerebrosidase; Chaperones; Gaucher Disease therapies; Lysosomal storage disorders.

Resumo

A doença de Gaucher é a doença de armazenamento lisossomal mais comum, podendo apresentar sintomas sistémicos e neurológicos. Esta é causada por mutações no gene *GBA* que codifica para a glucocerebrosidase (GBA), uma enzima envolvida na degradação de esfingolípidos nas células do lisossoma. A deficiência enzimática leva à acumulação do substrato de GBA nos lisossomas das células. A doença pode apresentar três tipos clínicos diferentes: o tipo 1 apresenta principalmente sintomas não-neuropáticos em múltiplos órgãos, enquanto que o tipo 2 e o tipo 3 são caracterizados por complicações neurológicas. Uma abordagem alternativa usa chaperonas farmacológicas para melhorar a atividade da enzima mutada e recuperar a sua atividade normal. Esta terapia tem o potencial de tratar os sintomas neurológicos, assim como os sintomas viscerais. Atualmente os únicos tratamentos disponíveis (terapia de substituição da enzima e terapia de redução do substrato) tratam os sintomas viscerais, mas não os sintomas neuropáticos

O principal objectivo desta tese é testar o efeito de oito compostos de chaperonas diferentes, na atividade e estabilidade da glucocerebrosidase. Estas chaperonas foram testadas em fibroblastos de pacientes com quatro génotipos diferentes da doença de Gaucher. Os resultados deste trabalho experimental sugerem que o efeito de uma dada chaperona depende da mutação que afecta a proteína GBA (glucocerebrosidase). As técnicas estabelecidas neste trabalho serão usadas, eventualmente, para testar o efeito dos compostos de chaperona em neurónios derivados de IPSc de pacientes com a doença de Gaucher, como potenciais agentes terapêuticos.

Palavras chave: Doença de Gaucher; Glucocerebrosidase; Chaperonas; Terapias para a doença de Gaucher; Doenças de acumulação lisosomal.

Resumo alargado

A doença de Gaucher é considerada uma patologia rara, no entanto tem vindo a atingir várias populações e famílias com uma taxa crescente, sendo uma doença que afecta adultos, crianças e bebés.

Esta patologia divide-se principalmente em três tipos: o tipo 1 que se caracteriza por afectar tanto crianças como adultos, podendo estes indivíduos viver apenas durante a sua infância ou eventualmente atingir a idade adulta. Uma das principais características que identificam o tipo 1 e diferem-no dos outros tipos da doença de Gaucher é o facto de o sistema nervoso central não ser afectado. O tipo 2 caracteriza-se por afectar maioritariamente crianças, possuindo uma esperança média de vida curta, tendo em conta que os indivíduos vivem em média cerca de nove meses. Ocorre no tipo 2, entre outras complicações, o envolvimento severo do sistema nervoso central. O tipo 3 afecta tanto crianças como adultos, sendo a esperança média de vida dos indivíduos o período da infância e, em alguns casos, a idade jovem adulta. No tipo 3 o sistema nervoso central está afectado, assim como no tipo 2. Existe ainda o tipo intermediário, entre o tipo 2 e o tipo 3, que se inicia com idade avançada, porém com rápida progressão de complicações neurológicas (Grabowski, 2008).

A presente doença é classificada como doença de depósito lisossómico, devido ao armazenamento que existe nos lisossomas das células intituladas células de Gaucher, onde decorre uma acumulação do substrato da enzima que sofre mutações. O gene *GBA* dá origem à enzima glucocerebrosidase, porém quando surgem mutações neste gene sucedem alterações no funcionamento normal da enzima. Atualmente estão referenciadas mais de 250 mutações neste gene, as quais contribuem para a atividade reduzida da enzima glucocerebrosidase. A anomalia na clivagem normal da enzima possibilita ao substrato desta acumular-se nos macrófagos, originando as denominadas células de Gaucher. Este armazenamento sucede principalmente no baço, fígado e medula óssea. Como resultado dessa acumulação, alguns órgãos são afectados provocando a doença de Gaucher (Grabowski, 2008).

A doença de Gaucher tem sido foco de investigação para métodos terapêuticos da mesma, uma vez que as terapias que existem atualmente não

possuem um alcance geral por parte de todos os pacientes, visto que as atuais formas de tratamento possuem um elevado custo e algumas delas não tratam completamente todos os tipos da doença. Assim, surge a necessidade de implementar novas terapias que possam combater as atuais limitações dos tratamentos convencionais para doença de Gaucher (Tropak et al., 2008).

Deste modo, sucede uma nova estratégia terapêutica para esta doença: um tratamento que utiliza chaperonas, proteínas que aumentam a atividade da glucocerebrosidase, a enzima mutada na doença de Gaucher. Este método inovador pretende aumentar a eficácia da terapia, melhorando os seus efeitos nos pacientes e alcançando todos os tipos existentes da doença. As chaperonas ajudam a estabilizar o *folding* da enzima mutada, aumentando e corrigindo o seu tráfego no retículo endoplasmático de forma a que exerça corretamente a sua função. Este processo tem sido investigado com o objectivo de melhorar as atuais condições dos pacientes da doença de Gaucher (Tropak et al., 2008).

É no âmbito da presente investigação na doença de Gaucher que se insere este trabalho experimental, o qual tem como objectivo testar os efeitos das chaperonas na atividade da glucocerebrosidase em fibroblastos de pacientes com diferentes genótipos.

Os fibroblastos de diferentes genótipos foram submetidos a um tratamento com as chaperonas medindo a concentração e a respectiva atividade da enzima glucocerebrosidase. Os resultados obtidos confirmam que em certos casos, as chaperonas utilizadas aumentam de facto a atividade da enzima mutada, revelando ser um método promissor para o tratamento da doença de Gaucher. O presente estudo demonstra ainda que o aumento sucede consoante a chaperona utilizada e a respectiva concentração da mesma. Existem também alterações devido aos diferentes genótipos das linhas celulares utilizadas neste estudo.

No futuro, as técnicas estabelecidas para medição da concentração e da atividade da glucocerebrosidade poderão ser utilizadas para medir os efeitos das chaperonas na enzima mutada em neurónios diferenciados de IPSc de pacientes com a doença de Gaucher. A fim de estabelecer o potencial desta opção terapêutica para a doença de Gaucher, particularmente para as suas formas neuropáticas.

Index of Abbreviations:

- 4MU	4-methyl-umbelliferone;
- aa	Amino acids;
- Ala	Alanine;
- Asn	Asparagine;
- bp	Base pairs;
- BSA	Bovine serum albumin;
- DMEM	Dulbecco's modified eagle's medium;
- ECL	Enhanced chemiluminescence reagent;
- EET	Enzyme enhancement therapy;
- ER	Endoplasmic reticulum;
- ERT	Enzyme replacement therapy;
- FBS	Fetal bovine serum;
- FS++	Immortal human fibroblast cell line used;
- GBA	Glucocerebrosidase;
- *GBA*	Gene *GBA;*
- GBAP	Pseudo gene *GBA*;
- GD	Gaucher Disease;
- GD1	1st Fibroblast cell line from GD patients (with type 2 GD);
- GD2	2nd Fibroblast cell line from GD patients (with type 2 GD);
- GD3	3rd Fibroblast cell line from GD patients (with type 2 GD);
- GD4	4th Fibroblast cell line from GD patients (with type 2 GD);

- GDT1	Gaucher disease type 1;
- GDT2	Gaucher disease type 2;
- GDT3	Gaucher disease type 3;
- Glu	Glutamic acid;
- GSL	Glycosphingolipids;
- HFF	Primary cell line from a human foreskin dissection used;
- hr	Hour(s);
- IPSc	Induced pluripotent stem cells;
- kb	Kilo basepair;
- Leu	Leucine;
- LU	Light units;
- min	Minutes;
- mRNA	Messenger RNA;
- nm	Nanometers;
-nmol	Nanomoles;
- PBS	Phosphate buffered saline;
- PC	Phosphate Citrate;
- PCR	Polymerase chain reaction;
- PCs	Pharmacological Chaperones;
- PE	Protein extract;
- Pro	Proline;
- PVDF	Polyvinylidene difluoride membrane;
- rpm	Rotation per minute;
- RT	Room temperature;
- Ser	Serine;

- SRT Substrate reduction therapy;

- TBST Tris-buffered saline and tween 20;

- WB Western blot;

- WT1 First wild-type cell line used;

- WT2 Second wild-type cell line used;

- μg Micrograms;

- μl Microliters;

- μM Micromolar.

Chapter 1

Introduction

1.1 Disease

Lysosomal storage disorders (LSD) are a group of rare diseases caused by accumulation of a diverse set of metabolic intermediates in cell lysosomes. Gaucher disease (GD) is a lysosomal storage disorder that can present both systemic and neurological symptoms and is inherited in an autosomal recessive way. It is the most common LSD, with a prevalence of approximately 1/75000 live births worldwide. However the disease presents higher prevalence in specific ethnic groups. GD is caused by mutations in the gene coding for glucocerebrosidase (GBA), enzyme that catalyses the breakdown of sphingolipids in the cell lysosome. As the result of this enzymatic deficiency, GBA substrates accumulate in the lysosome (Beck, 2007; Bohra & Nair, 2011; Butters, 2007; Vairo et al., 2013; Zhao, Keddache, Bailey, Gl, & Gaucher, 2003).

1.2 Historical Background

Gaucher disease was first described by the french physician Philippe Gaucher in 1882. In his doctoral thesis, Gaucher described a patient presenting progressive enlargement of the spleen (splenomegaly), nosebleeds, bruising, bleeding gums, weakness and weight loss. The spleen tissue presented an infiltration by large irregular shaped cells (now called Gaucher cells) that he noted under microscopic examination. Gaucher proposed that the woman suffered a rare form of spleen cancer (Brady, Kanfer, & Shapiro, 1965; Mehta, 2006). By 1904, it had been recognized that this was a distinct disorder that could run in families and affect both children and adults. Brill et al. described the familiar nature of GD (Brady et al., 1965; Mehta, 2006).

1.3 Clinical Presentation

Gaucher disease is a rare disorder in which patients can show a multisystemic clinical presentation, with a range of symptoms affecting a broad range of tissues and organs. Its symptoms can be broadly divided into systemic, affecting liver, spleen, bone marrow, lungs and skeletal system, and neurological, affecting the central nervous system (Figure 1)(Cassinerio, Graziadei, & Poggiali, 2013; G. A. Grabowski, Barnes, & Burrow, 2011; Gupta, Oppenheim, Kauvar, Tayebi, & Sidransky, 2011). Depending on the clinical presentation, Gaucher disease has been classically divided in three main types.

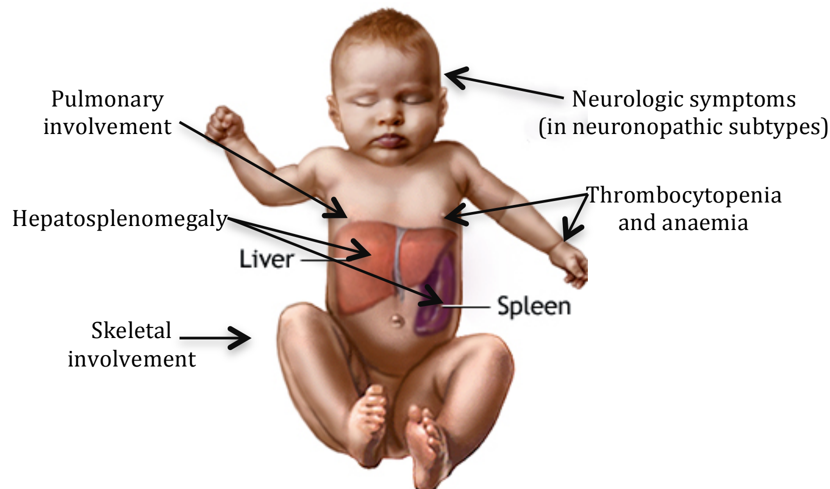


Figure 1 - Main symptoms observed in Gaucher Disease. *Adapted from:* "Hepatosplenomegaly: MedlinePlus Medical Encyclopedia Image," 2012.

1.3.1 Type 1

Gaucher disease type 1 (GDT1) is the most common form of the disease (~90% of Gaucher disease patients). Its onset is variable and can occur anywhere from 20 years of age and older. The typical major symptoms are hepatosplenomegaly, pancytopenia, leucopenia, bone loss, vertebrae colapse, pulmonar disease, cholelithiasis, cardiac and renal complications and secondary neurological complications related to bone disease (Figure 1). The most common

sign in type 1 variants is asymptomatic splenomegaly. Spleen volume can become 50 times bigger than the normal size, inducing early satiety and causing mechanical interference with respiratory function. Over 50% of the patients present hepatomegaly. In certain patients the liver can become massive, overflowing much of the abdomen. A majority of patients (75%-90%) have bone injuries, the most frequent being the Erlenmeyer flask deformity of the distal femur. Furthermore, destruction of the marrow cavity, lytic changes in bone cortex and osteoporosis/osteopenia may be observed. Osteopenia is a progressive process that is more suitable to be noticed in adulthood. There is a consistent absence of primary central nervous system disease in GDT1. Life expectancy for GDT1 patients is decreased (68 years vs. 77 years), regardless of gender (Cassinerio et al., 2013; G. A. Grabowski et al., 2011; Gupta et al., 2011).

1.3.2 Type 2

Gaucher disease type 2 (GDT2), also called 'neuronopathic' is the most severe form of the disease. Onset occurs shortly after birth with first symptoms occurring between 2-6 months of age. Typically, patients present growth arrest, bulbar and pyramidal signs, oculomotor abnormalities and severe neurological manifestations, including central nervous system symptoms such as spasticity, and eye paralysis. The cognitive involvement is general and usually starts as developmental stagnation, but later progresses to neurological degeneration. Mechanisms underlying neurodegeneration remain poorly understood. Patients gradually evolve microcephaly, stridor, trismus and epilepsy. Most children with Gaucher disease also develop hepatosplenomegaly, severe cachexia and joint contractures. Muscle-skeletal disease is normally not as evident in GDT2 as in other GD types. Patients eventually die of pneumonia or other infections, usually around 2 years of age. Severe cases show prenatal or perinatal symptoms. Prenatal symptoms include poor fetal movement, cardiomegaly, intrauterine growth retardation, non-immune hydrops fetalis, splenomegaly and hepatomegaly, and joint contractures or arthrogryposis (as a consequence of fetal akinesia). Approximately 35% of babies diagnosed perinatally with GDT2

are dysmorphic, with low-set ears, a small nose with a flat nasal bridge and hypertelorism (Cassinerio et al., 2013; G. A. Grabowski et al., 2011; Gupta et al., 2011). Fortunately, GDT2 is relatively rare (~10% of cases of Gaucher disease – 1:100 000 in the general population) (Cassinerio et al., 2013; G. A. Grabowski et al., 2011; Gupta et al., 2011).

1.3.3 Type 3

Gaucher disease type 3 presents both systemic and neurological symptoms. It is the chronic form of the disease, having an earlier onset than type 1, usually during childhood or early adulthood. The most common symptoms are hepatosplenomegaly, anaemia, thrombocytopenia, bone involvement, pulmonary infiltrates and esophageal varices associated with liver cirrhosis. The lifespan is severely reduced, with most patients dying in their 4th decade of life. There are three subgroups of type 3: type 3a – is characterized by progressive myoclonic epilepsy and dementia; type 3b - presents systemic disease and neurologic manifestations largely limited to horizontal supranuclear gaze palsy; and type 3c – presents neuropathic involvement with severe valvular and aortic arch calcifications (Cassinerio et al., 2013; G. A. Grabowski et al., 2011; Gupta et al., 2011).

1.3.4 Phenotypic continuum (an intermediate phenotype between type 2 and type 3)

There is an intermediate phenotype with onset later than usually identified in type 2, however with much severe neurological involvement than commonly observed in type 3. Signs and symptoms as horizontal supranuclear gaze palsy and refractory myoclonis seizures were described, as also as rapid decline due to these seizures in neuronal function with related ataxia, dementia and opisthotonus. Death occurs at 3 years old, caused by progressive brainstem involvement and aspiration pneumonia (Cassinerio et al., 2013; Goker-Alpan et al., 2003; G. A. Grabowski et al., 2011; Gupta et al., 2011).

1.4 Genetics

1.4.1 *GBA* gene

The *GBA* gene is located at chromosome 1q21, contains 11 exons and 10 introns and is approximately 7kb in length. The *GBA* gene location is represented in Figure 2. Alternative splicing results in multiple transcript variants. The region surrounding human *GBA* has been found to be particularly rich in genes, including seven genes and two pseudo-genes within a 85kb region of chromosome 1q. The putative TATA and CAAT like boxes of glucocerebrosidase promoter have been recognized about 260 bp upstream from the upstream ATG start codon (G. a Grabowski, 2012).

GBA

Figure 2 - *GBA* gene location on the long (q) arm of chromosome 1 at position 21. *Adapted from:* “GBA - glucosidase, beta, acid,” 2014; NCBI, 2014.

Several different mRNA species have been detected on Northern blots, which can be attributed to splice variants, alternate polyadenylation sites, or transcription of the pseudo-gene (G. a Grabowski, 2012).

The mRNA has 2324bp that translates into a 536-aminoacid protein Glucocerebrosidase. There are 3 isoforms of the protein. In normal conditions it is trafficked to the lysosome, where it catalyses the breakdown of glycolipid gluosylceramide to ceramide and glucose. The protein has four glycosylation sites at Asn58, Asn98, Asn185, and Asn309 and its catalytic residues are located at Glu274 and Glu379.

The tissue levels of *GBA* mRNA vary among different cell types, with high, moderate, low and negligible levels reported in epithelial, fibroblast, macrophage and B-cell lines, respectively (Hruska, LaMarca, Scott, & Sidransky, 2008).

1.4.2 *GBA* Pseudo-gene

There is a 5kb *GBA* pseudo-gene (*GBAP*) that is located approximately 16kb downstream from the *GBA* gene, on chromosome 1. *GBAP* is transcribed, an unusual characteristic for a pseudo-gene, but the open-reading frame is short due to several stop codons. Nonetheless, it has maintained a high degree of homology with the functional gene, sharing approximately 96% of the sequence in coding regions and the same organization of exons and introns. The different length of *GBA* and *GBAP* is explained by the presence of several *Alu* insertions in introns of *GBA.* The existence of this pseudo-gene is significant, as recombination events between *GBA* and its pseudo-gene can result in mutated *GBA* genes. Furthermore, the high degree of homology can complicate the identification of *GBA* mutations. The pseudo-gene carries a 55-bp deletion in exon 9, which may be of use for molecular diagnostic applications (Hruska et al., 2008).

1.4.3 Genotype – Phenotype

GBA carries out its function in the lysosome, the main site of cellular digestion. The complex molecules being digested can, ultimately, be external material phagocytized by the cell or can be constituted by cellular material destined for turn over. A complex catabolic network resides in the lysosome. Catalytic insufficiency on any one of the many enzymes involved in these catabolic pathways will result in accumulation of that enzyme's substrate, interruption of the metabolic pathway and indirect effects on other pathways. Glucocerebrosidase catalyses the breakdown of glucocerebroside into glucose and ceramide, and mutations in the *GBA* gene lead to Gaucher disease, characterized by glucocerebroside accumulation. The phenotype is essentially determined by the combination of mutations on both alleles, but environmental

factors may also play a role in the severity of the clinical presentation. *GBA* mutations are identified based on the amino acid position in the mature enzyme, with Ala40 designated as the first residue (G. A. Grabowski, 2008; Hruska et al., 2008). More than 250 different mutations in the *GBA* gene have been described, including point mutations, insertions and deletions (G. A. Grabowski et al., 2011; G. a Grabowski, 2012; Gupta et al., 2011; Lieberman, 2011). Recombination events between *GBA* and its highly homologous downstream pseudo-gene have produced a significant number of complex mutations; these can be the result of gene conversion, fusion or duplication. The 250 mutations described to date include 203 missense mutations, 18 nonsense mutations, 36 small insertions or deletions that lead to either frameshifts or in-frame alterations, 14 splice junction mutations, and 13 complex alleles resulting of recombination with *GBA* pseudo-gene. Some complex alleles can carry two or more mutations in *cis*. (Hruska et al., 2008).

A main point of interest in characterizing mutations for any genetic disease is the ability to predict clinical prognosis. In GD, we find a broad range of mutations causing a wide range of clinical presentations and a poor genotype-phenotype correlation. This makes it difficult to summarize our current knowledge on the subject; however, a number of observations can be made:

1. The most prevalent mutations are L444P (c1448T>C) and N370S (c.1226A>G).

2. The most common mutations in GD are: N370S, 84GG, L444P and IVS2+1.

3. The presence of the N370S mutation in one or both alleles is often associated to GDT1. The mutation is considered neuroprotective as individuals carrying this mutation rarely present neurological symptoms (Figure 3).

4. The presence of the mutations L444P or D409H is frequently associated to the development of neurological symptoms and frequently found in

GDT2 and GDT3 patients (Guggenbuhl, Grosbois, & Chalès, 2008; Tsuji et al., 1988) (Figure 3).

5. Many point mutations have been identified and homozygosity for those mutations has been associated with all types of GD.

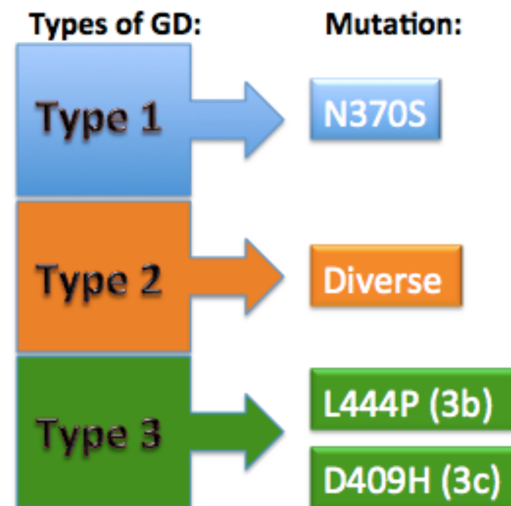


Figure 3 - Classification of Gaucher Disease on genotype-phenotype. Mutation N370S is one of the few recognized genotype-phenotype correlations related in patients with type 1 GD. In type 2 GD the L444P mutation is frequently found, however the recombinant and rare alleles are particularly predominant on this type. In type 3 GD patients commonly carry the L444P mutation and, as well, an atypical phenotype is related with mutation D409H on this type. *Adapted from:* Bohra & Nair, 2011; Hruska et al., 2008.

While it is possible to identify individual mutant alleles in GD patients, this capability has limited advantages, as it is difficult to predict phenotype from genotype. The majority *GBA* mutations can be found in all three types of GD patients.

Patients who share the same genotype may present different symptoms, clinical progress and responses to therapy. Conversely, patients with the same genotypes can show marked differences in clinical presentation. There are even distinct differences between siblings and twin brothers with the same genotype (Hruska et al., 2008; Park et al., 2003).

1.4.3.2 Gaucher Disease in specific ethnic groups

1.4.3.2.1 Ashkenazi Jews population

Ashkenazi Jews are the group with the highest prevalence of GD (1:855 live births). Usually, the carrier frequency for *GBA* mutations amongst this group is set between 1 in 14 and 1 in 18. The most frequent mutation is the A->G transition at 1226G, the mutation that causes an Asn->Ser substitution at amino acid 370 of the mature protein. Mutation N370S accounts for approximately 70% of mutant alleles (Sidransky, 2013). This mutation is present in approximately 6% of the Ashkenazi Jews population and is the main cause of the high incidence of Gaucher disease in this ethnic group. The second most frequent mutation is an insertion of a guanine at nt.84 of the cDNA and has an incidence of about 0.6%. Among this population, the combined frequency of the N370S and c.84dupG mutations is 0.0343, resulting in a combined incidence of 1:855. This population generally presents type 1 disease (Sidransky, 2013).

1.4.3.2. Norrbottinian population

In the Norrbottinian population, in Northern Sweeden, there is an AT → C transition at nt.1448, which produces a Leu → Pro substitution at amino acid 444 of the mature protein. It has a moderately high frequency amongst this population even though it is revealed in other populations at lower frequencies. This population commonly presents type 3 GD (Sidransky, 2013).

1.4.3.3. Genotype analysis

A number of PCR based genotyping assays are available for the most frequent GD mutations, but the identification of mutant *GBA* alleles can be problematic, as primers must be designed to discriminate between the functional gene and the pseudo-gene (Hruska et al., 2008). Furthermore, detection of

recombinant alleles can pose particular difficulties. Southern blot can help resolve ambiguities, but ultimately, direct sequencing is being increasingly used to characterize mutant alleles, as this technique can detect structure as well as frequent, rare or novel mutations (Hruska et al., 2008).

1.4.3.4. Inheritance patterns of the disease

GD inheritance is autosomal recessive. Heterozygotes are generally asymptomatic. Assuming both parents are heterozygotes, each child of the couple has a 25% probability of being affected, a 50% chance of being an asymptomatic carrier and a 25% possibility of not carrying a GD allele (G. a Grabowski, 2012; Pastores & Hughes, 2014). Prenatal diagnosis may be offered to high-risk families and the diagnosis consists on glucocerebrosidase assays in amniotic fluid cells or chorionic villi. Based on this information and the identification of *GBA* or *GBAP* mutations in family members, an expected phenotype can be deduced (Guggenbuhl et al., 2008).

1.5 Molecular mechanisms of GD

1.5.1 Sphingolipid metabolism

Sphingolipids are synthesized in the endoplasmic reticulum from nonsphingolipid precursors. Sphingolipids perform important functions in membrane biology and therefore have important effects on cell metabolism. Although there is a great diversity on both structure and function of sphingolipids, all of them share a common anabolic and catabolic pathway (Gault, Obeid, & Hannun, 2011). The metabolism of sphingolipids can be viewed as a set of interconnected networks that diverge from a single common entry point and flock to a single common breakdown pathway. Their simplest forms – sphingosine, phytosphingosine or dihydrosphingosine – serve as the backbone of more complex molecules. For example, the acylation of sphingosine, phytosphingosine or dihydrosphingosine with a variety of acetyl-CoA molecules by the action of various ceramide synthase gives rise to long molecules such as

ceramide or phytoceramida dihydriceramida (Gault et al., 2011). A particular class of sphingolipids are the glycosphingolipids (GSL) that are characterized by the kind of sugar residues attached to their ceramide headgroup. GSL are broadly divided into two categories: glycosphingolipids and galactosphingolipids (Gault et al., 2011).

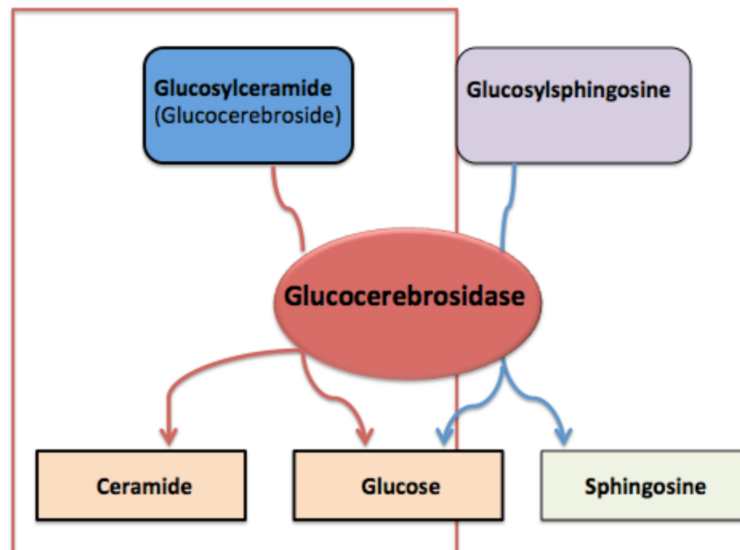


Figure 4 - Glucocerebrosidase function. Glucosylceramide is cleaved by Glucocerebrosidase into Ceramide and Glucose, in the normal function of the enzyme, as represented in the red square. Whereas Glucosylsphingosine is cleaved by Glucocerebrosidase into Glucose and Sphingosine. Glucocerebrosidase is mutated in GD resulting in the accumulation of Glucosylceramide mainly in the lysosomes. *Adapted from*: Sidransky, 2013.

1.5.2 Molecular mechanisms

The enzyme glucocerebrosidase (also known as GBA; acid beta-glucosidase; beta-glucocerebrosidase; glucosylhydrolase; glucosylceramidase) is synthesized in the ER and trafficked to the lysosome. While on route, it suffers post-translational modifications, mainly in the form of addition of mannose

carbohydrates at selected Asn residues. Once in the lysosome, it hydrolyses the glucocerebroside (also called glucosylceramide) to glucose and ceramide, as shown in Figure 4. As a direct result of mutation, enzyme structure is altered with several possible consequences, depending on the nature of the mutation. A mutation in the active site of the enzyme will affect its catalytic function. A mutation elsewhere in the enzyme can also affect catalytic activity indirectly. Another consequence of a mutation can be a modification of the stability of the protein, resulting in increased protein degradation, which ultimately diminishes the amount of active enzyme reaching the lysosome. As a result, the enzyme's substrate, glucosylceramide is accumulated. In monocyte-lineage cells, these lipid-laden lysosomes result into the characteristic "Gaucher cells". They present a large reticuloendothelial system, a small and eccentrically placed nuclei and a striated cytoplasm as shown in Figure 5 (G. A. Grabowski, 2008; Vairo et al., 2013).

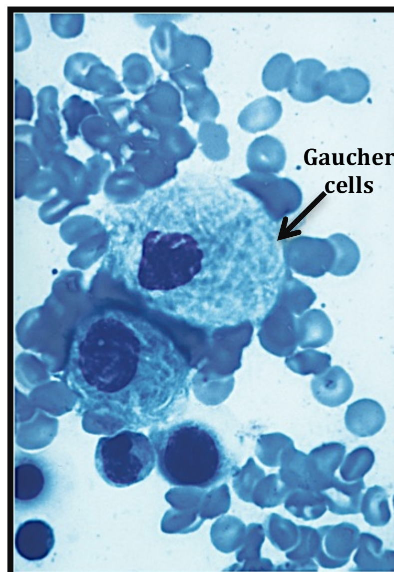


Figure 5 - Gaucher cells from a bone marrow aspirate. These cells derive from macrophages with stored glycolipids. They measure around 20 – 100μm in diameter. Gaucher cells are also swollen and have a cytoplasm that looks lacy. *Adapted from:* Elstein, Abrahamov, Hadas-halpern, & Zimran, 2001; Vairo et al., 2013.

The dysfunctional catabolism of glycosylceramide and the enlargement of macrophages by glucocerebrosidase substrates accumulation are the basis of the visceral manifestations in GD. Central to the multisystemic presentation of the disease is the fact that monocytic cells in circulation penetrate multiple organs and differentiate into macrophages. Mouse models of GD have shown that the classic and alternative pathways of macrophage activation are both modified in the GD. However the mechanisms whereby systemic and organ-specific involvement are initiated and developed remain poorly understood (G. A. Grabowski, 2008). Another process that may result from abnormal folding of mutant proteins in the endoplasmic reticulum is triggering of apoptotic or inflammatory responses in several tissues. Therefore the development of agents capable of neutralizing the unfolded protein response could be an effective therapeutic strategy in GD (G. A. Grabowski, 2008).

1.6 Therapeutic Approaches

In any disease the goal of therapy is to improve the patients quality of life by either healing the disorder or managing its outcome. Numerous treatments and approaches are in use or under development for GD. All of them are intended to reduce glucosylceramide accumulation and to minimize the consequences of its storage in patients. Current treatments only address the systemic aspects of the disease (Gupta et al., 2011; Lieberman, 2011).

<u>Therapeutic Approaches to GD treatment:</u>

- Enzyme replacement therapy (ERT);
- Substrate reduction therapy (SRT);
- Bone marrow transplantation (also known as stem cell transplantation);
- Surgical (splenectomy).

1.6.1 Enzyme replacement therapy

Enzyme replacement therapy (imiglucerase) uses recombinant enzyme administered intravenously to provide functional glucocerebrosidase to patients. The first GD therapy used was based on an alglucerase (Ceredase®), approved by FDA in April 1991. In May 1994 FDA approved imiglucerase (Cerezyme®), which replaced the use of alglucerase (Gupta et al., 2011; Lieberman, 2011).

Infused enzyme is endocytosed by circulating macrophages and eventually reaches the lysosomal compartment where it restores function. As a result, there is a significant decrease in size of the liver and spleen, a reduction in bone abnormalities and also some reversion in other typical GD clinical manifestations. However pulmonary symptoms respond poorly. Due to the costs of recombinant enzyme production and the relatively small market, this therapy is expensive for patients and health care systems, amounting to over 200,000€ per patient per year. Other drawbacks of ERT therapy involve the inconvenience of regular infusions, risk of side effects and immunological rejection to the recombinant enzyme (Gupta et al., 2011; Tiscornia et al., 2013).

1.6.2 Substrate reduction therapy

Substrate reduction therapy uses a small molecule inhibitor of the glucosylceramide biosynthetic enzyme. The main goal of this strategy is to inhibit synthesis of glusosylceramide. If the substrate is not synthesized in the first place, it cannot accumulate if glucocerebrosidase is absent. This method uses an imino sugar as inhibitor (Miglustat®) of the first step in glycolipid synthesis. Currently the only FDA-approved SRT agent is *N*-butyl-deoxynojirimycin (NB-DNJ) (Miglustat or Zavesca®), which is mainly used to treat GD type 1 patients. However, this treatment is also expensive and presents adverse reactions (non allergic) (Gupta et al., 2011).

1.6.3 Bone marrow transplantation (stem cell transplantation)

Bone marrow transplantation, also known as stem cell transplantation is one alternative treatment for Gaucher disease. It has a lower cost and the patients' response has been positive. This treatment has been used to treat GD non-neurological manifestations, since the transplanted bone marrow restores a population of monocytes with active glucocerebrosidase. For unclear reasons, this procedure seems to be an appropriate option to treat GD type 3 patients. The approach still has significant risks associated, particularly a 10% incidence of mortality due to surgery (Gupta et al., 2011).

1.6.4 Surgical intervention

Splenectomy (surgery to remove spleen) can be partial or total and may be an option in exceptional cases if the organ acquires dimensions that affect the patient's daily quality of life, or if it has significant areas of infarction. In certain cases, as when the patients suffer from severe thrombocytopenia, present high risk of bleeding or anaemia, surgical interventions are not recommended. Partial splenectomy can achieve the benefits of complete splenectomy and reduces the risk of infection. However, re-growth of the splenic remnant has been observed, along with the re-emergence of pre-operative symptoms and new bone involvement can occur after most of partial splenectomies (Gupta et al., 2011).

1.7 Pharmacological Chaperones (PCs)

1.7.1 Chaperone therapy

It remains a challenge to find a therapy to stop, stabilize or reduce the neurological manifestations of the disease in GDT2 and GDT3. ERT has no effect on CNS symptoms, mostly due to the inability of the recombinant enzyme to cross the blood-brain barrier. The response to SRT is also poor. The identification of prenatal lethal forms show that the neurological damage may

already be present in the most severe forms of the disease, adding a further barrier to the therapeutic agent (Gupta et al., 2011).

In recent years a novel therapeutic approach is being researched and developed in GD and other LSD, involves the use of pharmacological chaperones (PC), or enzyme enhancement therapy (EET). PCs are small organic molecules capable of reversible interaction with glucocerebrosidase in such a way that they restore the original 3D structure of the protein or otherwise stabilize its conformation. The use of PCs stabilizes the native conformation of mutant enzymes as they fold in the endoplasmic reticulum, enabling them to pass the ER quality control system and preventing the ER associated degradation system. Therefore it enables their trafficking from the endoplasmic reticulum to the Golgi and to the lysosome. Once the chaperone-enzyme complex reaches the lysosome, the chaperone-enzyme complex dissociates due to presence of excess substrate. Some strategies for synthesis attempt to design PCs that are sensitive to pH, having high affinity of the enzyme at neutral pH (as is found in the ER) but low affinity at lower pH (as found in the lysosome) (Tropak et al., 2008).

PCs represent a particularly promising therapeutic strategy because they combine the benefits of a small-molecule approach-oral bioavailability, cell permeability and the capacity to cross the blood-brain barrier with the specificity and selectivity of enzyme replacement therapy. These small molecules can be less expensive than other therapies such as ERT and offer the possibility of treating the neurological aspects of type 2 and 3 GD patients (Tiscornia et al., 2013; Tropak et al., 2008; Yu, Sawkar, Whalen, Wong, & Kelly, 2007). Recent studies report that PCs have good properties regarding oral administration, specifically enhancing glucocerebrosidase activity in mouse tissues as the brain and also lacking acute toxicity at high doses in normal mice. It is generally accepted that even small increases in glucocerebrosidase activity would be required to reach a clinical effect, therefore chaperone therapy results warrants further development (Tiscornia et al., 2013). Moreover, the use of a fluorescent labelled derivative demonstrated its ability to cross the cell membrane and increased the levels of glucocerebrosidase in mature and also immature neuronal cells (Tiscornia et al., 2013). Several promising chaperone candidates for GD are currently in in phase I and II of clinical trials (Gupta et al., 2011).

1.8 Objective

The main goal of this thesis is to test 8 different novel PCs in terms of their effects on glucocerebrosidase stability and activity in fibroblast primary cultures from GD patients with 4 different genotypes. This approach is an important first step in characterizing these novel pharmacological chaperone candidates. We therefore set out to establish several techniques needed for measuring protein levels and enzymatic activity of GBA in our laboratory. In the long term, these techniques will be used to test the effect of chaperone compounds on neurons derived from IPSc of GD patients, to determine potential uses in therapy.

Chapter 2

Materials and Methods

2.1 Cell harvest and protein extraction

1. The cells were washed twice with PBS 1x and harvested by trypsinization or scrapping. Cells were centrifuged for 5min at 3000rpm and all cell pellets were frozen and stored at -20ºC.
2. RIPA buffer was added and the cell pellet resuspended.
3. The cell suspension was incubated on ice for 15 min mixing every 3 min.
4. The lysate was centrifuged at 13,000 rpm at 4ºC for 15 min.
5. The supernatant was collected, aliquoted and stored at -20ºC.

For reagent preparation: see Annexes.

2.2 Protein Concentration Assay

Protein concentration was determined by the Bradford method:

1. Using a stock solution of BSA (10µg/µl), the following standard curve was prepared in triplicate:

Final concentration	Stock solution volume	Water volume used to dilute stock solution
BSA µg/µl	**µl BSA 10µg/µl**	**µl H20**
0	0	100
0.2	2	98
0.4	4	96
0.6	6	94
0.8	8	92
1	10	90

2. 1 µl of each standard was added to 99 µl of Bradford reagent.

3. Samples were diluted between 1/1 and 1/100, as required, until the reading fell into the linear range of the standard curve.

4. Absorbance at 595nm was determined and plotted against BSA concentration. The slope of the line (m) was calculated. To calculate protein concentration, the absorbance at 595nm for each sample was divided by m and multiplied by the dilution factor, giving protein concentration in μg/μl.

Preparation of reagents: see Annexes.

2.3 Glucocerebrosidase Enzymatic Activity Assay

1. Protein extracts were thawed on ice;

2. Using a stock solution of 4-methyl-umbelliferone (SIGMA® M1381) (1.42nmol/μl), a standard curve was prepared in triplicate according to the following table:

Stock solution 1.42 nmol 4MU/μl	**PC buffer**	**Working solution 4MU**
μl	**μl**	**nmol**
0	20	0
1	19	1.42
2	18	2.84
5	15	7.19
10	10	14.2
15	5	21.3
20	0	28.4

3. 200μl of Glycine buffer were added.

4. Samples were subjected to fluorometry by exciting at 366nm and reading absorbance at 446nm. Emission was plotted against nmol of 4MU and the slope of the line (m) determined.

5. For each sample, dilutions were made between 1/1 and 1/100, as necessary, until the reading fell into the linear range of the standard curve.

6. 10μl of PE for each sample (in triplicate) were mixed with 20μl of Substrate Solution and incubated at 37ºC for 1 hour. Reaction was stopped by addition of 200μl of Glycine Buffer.

7. Samples were subjected to fluorometry by exciting at 366nm and reading emission at 446 nm. Emission (light units) was plotted against nmol of 4MU.

8. The light unit value for the sample was divided by (m) to obtain nmol of MU produced by GBA present in the 10μl of sample tested. This value was normalized to total protein in the sample tested and expressed as nmol of 4MU/μg protein/ hour.

2.4 Protein Expression

2.4.1 Western Blot:

1. A 10% resolving gel of polyacrylamide/ bis acrylamide 29:1 and stacking gel were prepared;
2. 10-100 μg of PE were loaded per lane;
3. The gel was run at 130V for 1 hour;
4. Resolved proteins were transfered onto a PVDF membrane at 20V for 1 hour;
5. Membrane was incubated in blocking buffer for 1 hour;
6. Membrane was incubated in primary antibody, overnight at 4ºC;
7. Membrane was washed 3 times in TBST (1x) and incubated in secondary antibody for 1 hour at room temperature;
8. Membrane was washed 3 times in TBST (1x);
9. Membrane was developed with 5ml ECL reagent.

Preparation of reagents and buffers: see Annexes.

2.4.2 Antibodies used in this study:

AntiGBA: Mouse monoclonal to GBA 1x100 ug; abcam®discovermore; Ref.: 798203747835; Product: ab55080; Dilution: 6ul AB in 6ml BSA blocking solution.

Goat anti-mouse IgG peroxidase-conjugated 0,8 mg/ml; Affinipure®; 115-035-003; Dilution: 1ul AB in 10ml BSA blocking solution.

AntiActin: Actin (I-19): sc-1616 - Actin goat polyclonal; Santa Cruz Biotechnology, Inc®; starting dilution 1:200, dilution range 1:100 - 1:1000; diluted in BSA blocking solution.

Goat anti mouse IgG-HRP: sc-2005; Santa Cruz Biotechnology, Inc ®; dilution: 1:5000; diluted in BSA blocking solution.

2.5 Chaperone treatment

Chaperones

The pharmacological chaperones were synthesized and characterized by a collaborator group, who provided them number coded as stock solutions. Chaperones were labelled from 1 to 8.

WT and GD fibroblasts cultured in DMEM + 10% FBS at 37ºC. If required cells were cultured in the presence of different concentrations of pharmacological chaperones and the medium was changed every 48hs. Typically cultures were exposed to chaperone treatment for 6 days before harvesting cells for analysis.

Cell lines used in this study:

WT1: an immortalized human fibroblast cell line.

WT2: a primary cell line from a human foreskin dissection.

Fibroblasts from GD patients were received from a collaborator group and had the following genotypes, passage and patient age. All patients were diagnosed as GD type 2 (GDT2).

Name	Genotype	Passage	Patient age
GD1	N188S/ G193W	P2	30 Years
GD2	F213I/ RecNaI	P1	3 Years
GD3	L444P/ L444P	P1	9 Years
GD4	L444P/ R120W	P1	1 Month

Chapter 3

Results and Discussion

3.1 Initial characterization of cell lines used in this study:

The fibroblast populations used in this study consisted of 4 primary cultures from 4 patients diagnosed with GDT2 with 4 different genotypes. In order to make an initial characterization of these GD fibroblast populations, we proceeded to determine the levels of GBA protein and activity in comparison with two control fibroblast populations by Western Blot (WB) and fluorometry. Two fibroblast populations that were wild type (WT) in terms of GD mutations were used as negative controls (WT1 and WT2). More details and characteristics on these cell lines can be found in the section Materials and Methods. This experiment also had the goal of setting up the protocols to be used in both WB and GBA fluorometry assays, which had not been previously done in our laboratory.

3.2 Characterization of Glucocerebrosidase activity in the cell lines used in this study.

In order to measure glucocerebrosidase activity, it was firstly necessary to determine the protein concentration of the samples.

3.2.1 Bradford Assay:

Cells were cultured, harvested and protein extracts made as indicated in Materials and Methods. We then determined protein concentration using the Bradford method. This method is a colorimetric assay based on a colour shift that occurs when the dye Coomasie Brilliant Blue (brown) binds to proteins forming a complex (blue) that can be quantified by measuring absorbance at 595 nm. When the Bradford reagent is added to a solution containing protein, the protein-dye complex forms and the intensity of the blue colour will be

proportional to the concentration of protein. In order to correlate colour intensity with the amount of protein, a standard curve is created. Using a protein solution of known concentration, a serial dilution is made, aliquots mixed with the Bradford reagent, and absorbance at 595 nm measured. When absorbance is plotted against protein concentration, absorbance increases in a linear way as protein concentration increases, until absorbance reaches a plateau of saturation, after which presence of higher amounts of protein do not result in increased absorbance. The linear range of the curve is used to determine the protein concentration in unknown protein samples. The standard curve is shown in Figure 6.

Panel 1:

BSA ug/ul	Abs.@595nm
0	0
0,02	0,004
0,2	0,019
2	0,119

Panel 2:

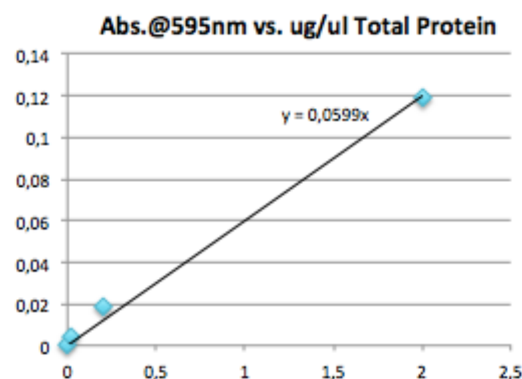


Figure 6 - BSA absorbance standard curve determined by spectrophotometry. Panel 1: Table showing concentration of BSA used [BSA](μg/μl) and the corresponding mean 595nm absorbance values for each dilution; Panel 2: resulting standard curve; four different BSA concentrations were used to determine the standard curve, with technical triplicates were performed for each point.

We then proceeded to determine protein concentration in our WT and GD cell lysates (Figure 7). To do so, we performed the Bradford assay on aliquots of our protein extracts. We found that the concentration of protein in samples WT1 and WT2 was higher than that of the GD samples; therefore we diluted the WT samples 1/5 and 1/10 in order to obtain 595nm readings that would fall within the linear range of the standard curve.

Abs. @ 595nm			
Protein Samples	Undiluted	Diluted 1/5	Diluted 1/10
WT1		0,016	0,048
WT2	0,121	0,037	
GD1	0,089	0,028	
GD2	0,089	0,02	
GD3	0,091	0,026	
GD4	0,101	0,029	

Figure 7 - Absorbance of protein extracts of WT and GD protein extract samples. Undiluted or diluted samples were measured for absorbance at 595nm by spectrophotometry.

Using the absorbance values, we calculated the protein concentration of the WT and GD protein samples. Results are shown in Figure 8.

Samples	Abs. @ 595nm	Dilution	Protein Concentration (ug/ul)
WT1	0,048	1 in 10	8,0
WT2	0,037	1 in 5	3,1
GD1	0,089	none	1,5
GD2	0,089	none	1,5
GD3	0,091	none	1,5
GD4	0,101	none	1,7

Figure 8 - Protein extract concentration from WT and GD patients. Using the values of absorbance at 595nm obtained and the Bradford standard curve, protein concentration was calculated in μg/μl (taking into account dilution) for each sample.

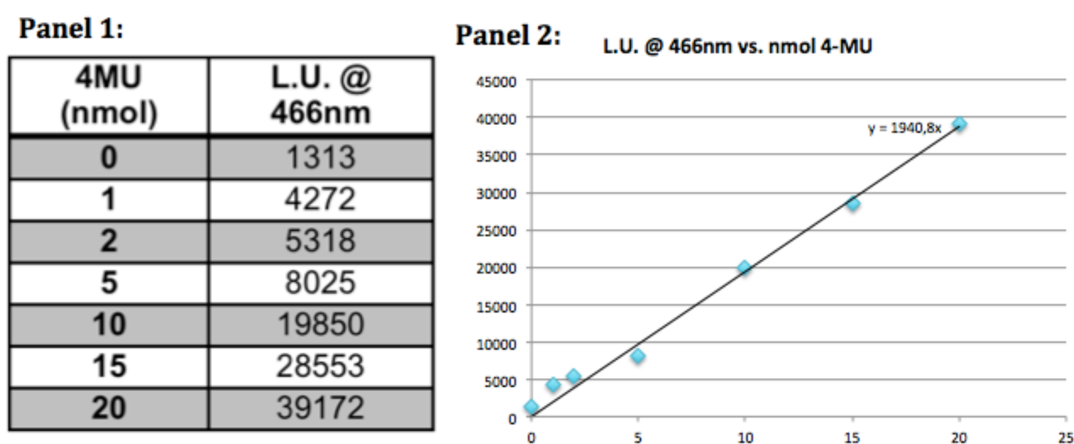
3.2.2 Glucocerebrosidase Assay:

In order to quantify the amount of glucocerebrosidase present in a given sample, aliquots of the protein extracts from the samples were mixed with non-fluorescent substrate of glucocerebrosidase called 4-methylumbelliferyl-B-D-galactoside (4-MU-gal). Glucocerebosidase catalyzes the degradation of substrate into the fluorescent product 4-methylumbelliferone (4-MU), as represented in Figure 9.

Figure 9 - GBA enzymatic activity assay scheme. GBA (Glucocerebrosidase) catalyzes the degradation of the non fluorescent substrate 4-methylumbelliferyl-B-D-galactoside into the fluorescent product 4methylumbelliferone.

The resulting product, 4-methylumbelliferone (4MU) can be quantified by fluorescence by exciting at 366nm and measuring light units of emission at 446nm. Aliquots of protein extracts were mixed with 4-MU-gal substrate solution, incubated for 1 hour and the reaction slopped by lowering the pH by addition of excess volume of Glycine buffer (pH = 2). The quantity of light units can therefore measure the fluorescent product resulting from the reaction catalysed by glucocerebrosidase and once the substrate is in excess, the fluorescence will be proportional to the amount of active enzyme present in the protein extracts. In order to correlate light units measured with the amount of 4-MU obtained, we created a standard curve shown in Figure 10 panel 2. Using a 4-MU solution of known concentration, a serial dilution was made to create aliquots with known amounts of 4-MU, excited at 366nm and emission at 446 nm

was measured (Figure 10 panel 1). When light units are plotted against protein concentration, light units increase in a linear way as 4-MU amount increases, until emission reaches a plateau of saturation, after which presence of higher amounts of 4-MU do not result in increased emission. The linear range of the curve is used to determine the amount of 4-MU corresponding to a given number of light units.



Panel 1:

4MU (nmol)	L.U. @ 466nm
0	1313
1	4272
2	5318
5	8025
10	19850
15	28553
20	39172

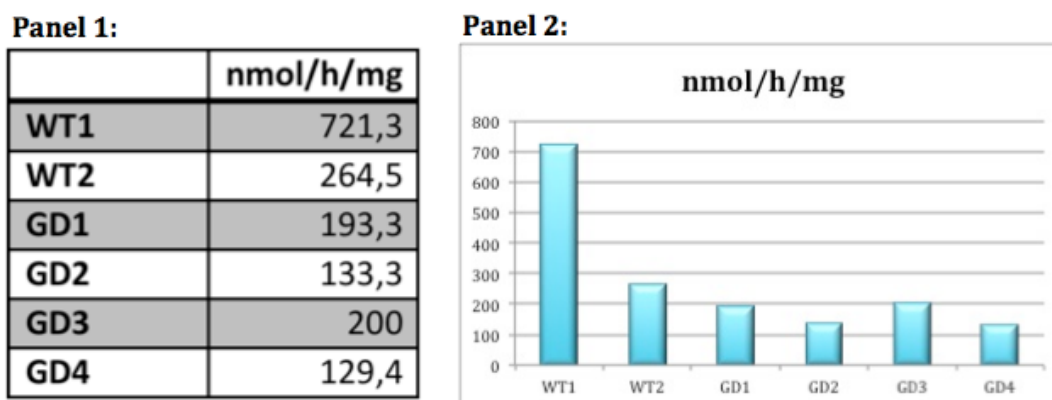
Figure 10 - 4MU (4 methylumbelliferone) fluorescence standard curve determined by fluorometry. Panel 1: 4MU (nmol) and the corresponding mean light unit values. Panel 2: 4-MU standard curve (six different amounts of 4MU (nmol) were used to determine the standard curve and technical triplicates were performed for each point) The Y axis corresponds to light units and the X axis corresponds to nmol of 4-MU.

We then proceeded to perform the glucocerebrosidase assay on 10 μl of our WT and GD protein samples. Since the protein extract of sample WT1 had a high concentration, there was a need to dilute the sample 10 times to bring it into the linear range of the standard curve. Using the standard curve presented in Figure 10 panel 2, we determined the nmol of 4-MU present in a 10 μl aliquot of each of our protein extracts (Figure 11).

	L.U. @ 466nm	Dilution	4-MU (nmol)
WT1	11208	1 in 10	57,7
WT2	15991	-	8,2
GD1	5675	-	2,9
GD2	3836	-	2,0
GD3	5869	-	3,0
GD4	4224	-	2,2

Figure 11 - Glucocerebrosidase assay on protein extracts of WT and GD patient samples. 10 μl aliquots of protein extracts from WT and GD patient fibroblasts were assayed and light units (LU) determined by exciting at 366nm and measuring emission at 446nm. The standard curve was used to determine the amount of 4-MU present in the aliquot of each sample.

After having determined a) the amount of 4-MU present in each sample and b) the concentration of total protein in the extracts of each sample, we proceeded to calculate the corresponding glucocerebrosidase activity in each sample. With the amount of 4-MU produced by each extract in 1 hour of reaction, expressed in relation to unit of protein, we could obtain a measure of glucocerebrosidase activity expressed as nmol of 4-MU/hr/mg of total protein (Figure 12, Panel 1). These values are shown as a histogram (Figure 12, Panel 2).



Panel 1:

	nmol/h/mg
WT1	721,3
WT2	264,5
GD1	193,3
GD2	133,3
GD3	200
GD4	129,4

Figure 12 - Glucocerebrosidase activity expressed as nmol 4-MU/hr/mg total protein. Panel1: GBA activity of WT and GD samples. Panel 2: values shown in a histogram chart.

Due to the fact of being a preliminary experiment and there is a lack of technical replicates on this GBA activity assay we are not able to show error bars. Also, the level of GBA activity obtained for sample WT2 is different from what we were expecting. Results in the literature indicate the level of GBA activity in GD samples can vary, depending on tissue or cell type and on genotype. Generally, GBA activity can range approximately between 1% and 20% of the levels found in WT samples. Therefore, the results shown in Figure 12, Panel 2 are in line with what we expected, with the exception of sample WT2, which for unknown reasons seemed to have a significantly lower GBA activity than would be expected for a WT cell line. WT2 fibroblasts were obtained from a foreskin biopsy of a healthy person. One possible explanation for the low GBA level of this person is that he might be an undetected carrier of a mutated *GBA* allele. If this were the case, he would not show signs of disease and his GBA levels would be expected to be 50% of those found in a person with no mutations in *GBA*. Testing for this hypothesis would require testing for common GD mutations by PCR or sequencing the *GBA* gene. We did not pursue this course of action. If sample WT2 is considered 'anomalous' and only WT1 is taken into further analysis, the results are what we expected: high level of activity in WT1 and uniformly low levels of activity in all 4 GD samples.

In order to extend our characterization, we proceeded to determine the levels of glucocerebrosidase protein in our samples by Western Blot analysis.

3.2.3 Western Blot Analysis

The protein extracts of our WT and GD samples were loaded on a 10% polyacrylamide bis acrylamide gel, subjected to electrophoresis and transferred to a PVDF membrane as described in Materials and Methods. Results are shown in Figure 13.

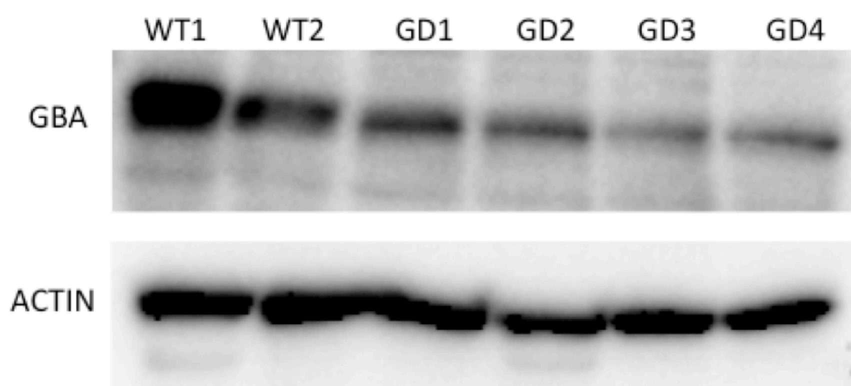


Figure 13 - Western Blot of WT and GD protein samples. Samples were loaded on a 10% polyacrylamide bis acrylamide gel (30 μg/lane), electrophoresed, and transferred to a PVDF membrane. Blocking was done in 5% BSA for 1 hr at RT. GBA: Primary antibody was AntiGBA (Affinipure ®) diluted in blocking solution overnight at 4ºC. After washing, secondary antibody was AntiGBA (abcam®discovermore) diluted in blocking solution for 1 hr at RT. Actin: Primary antibody was AntiActin (SC-1616 Santa Cruz Biotechnology®) diluted in blocking solution overnight at 4ºC. After washing, secondary antibody was AntiActin (SC-2005 Santa Cruz Biotechnology®) diluted in blocking solution for 1 hr at RT.

The results shown in Figure 13 are consistent with those corresponding to glucocerebrosidase activity shown in Figure 12. Actin levels indicate that all lanes contained similar amounts of protein. The relatively weak signal observed for GBA in WT2 on the western blot, is consistent with the low GBA activity observed in the GBA activity assay, suggesting indeed that the low enzymatic activity is a result of low glucocerebrosidase concentration in that sample. The strong consistence between the results of the GBA activity assay and the western blot analysis reinforces the conclusion that the cell lines used in this study, with the exception of WT2, behave as expected in terms of glucocerebrosidase levels and activity.

3.3 Effect of chaperone compounds on glucocerebrosidse activity levels in fibroblasts from 4 GD patients with different phenotypes:

As described above, we were able to establish GBA fluorometric assays in our laboratory. Before proceeding to the actual experimental measurements that we proposed to do, we ran the GBA fluorometric assay several times on only one GD line treated with only one chaperone compound in order to fine-tune some of the experimental details. It was of extreme importance to optimize that technique in order to obtain accurate measurements. In particular, we wanted results from different experiments to be comparable, so it was important to establish a standardized procedure for the assay and particularly to process the resulting data. By trial and error, we developed a detailed step-by-step protocol for assay and data treatment:

1) Typically, an experiment would involve a chosen GD fibroblast line. Cells would be plated and treated with different concentrations of a given chaperone compound. Cells cultured in absence of the chaperone constituted the negative control.
2) Cell cultures were observed and any toxicity effects caused by the chaperone treatment noted.

3) Protein extracts were made and protein concentration determined by Bradford assay as described in Materials and Methods.
4) For the GBA flurometric assay, each sample was tested in triplicate.
5) Each 96 well plate included 3 wells with all reagents except protein extracts (background fluorescence control).
6) Each 96 well plate included a GBA assay standard curve. Each point of the curve was measured in triplicate.
7) Once reading was performed, fluorescence values (light units) for each triplicate reading were examined and values that are obviously wrong (i.e., more that 2 standard deviations from the mean) eliminated from the analysis.
8) The average of the 3 readings of background fluorescence were calculated.
9) The value of background fluorescence was substracted from every individual measurement.
10) The triplicate measurements for every point of the standard curve were averaged, the standard curve plotted and the slope of the curve (m) calculated.
11) For each measurement, GBA activity was calculated (μg 4MU/hr).
12) Each activity value was normalized to total amount of protein (μg 4-MU/hr/mg).
13) Average GBA activity values and standard deviations for each treatment were calculated and plotted as a histogram, with the activity level of the control (no chaperone treatment set to an arbitrary value of 100).

Once this procedure was established, we began measuring the effect of each chaperone on each of the GD lines. To do so, for each of the GD lines (GD1, GD2, GD3 and GD4), cultures were seeded and cultured for 6 days with each of the 8 chaperone compounds (1, 2, 3, 4, 5, 6, 7 and 8) at concentrations of 0, 5, 15, 30 and 50 μM. Media was changed every 2 days.

3.3.1 Effect of 8 chaperone compounds on GD1 fibroblasts (GDT2 genotype N188S/G193W):

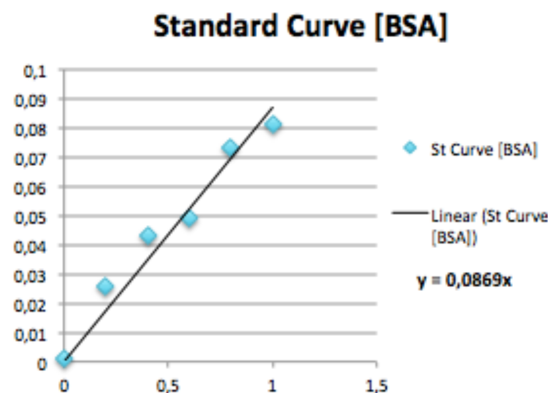
In this section, we will present the detailed 13th step procedure outlined above as applied to GD1.

Cells were seeded and treated with chaperone compounds as described above (Step 1). No cytotoxic effects were observed (Step 2). Protein extracts were made and protein concentration determined by Bradford (Step 3). Results are presented in Figure 14. Interestingly, protein concentrations were overall similar for all samples, mostly ranging between 0,6 and 0,9 μg/μl. We did notice slightly lower values for the 50μM concentration of chaperones #4, 5 and 6, possibly suggesting low level cytotoxicity with these concentrations (despite no morphological alterations had been observed). The GBA assay standard curve and GBA activity determination of the protein extracts was performed in triplicate (Steps 4, 5 and 6). Once the measurement was made, anomalous readings were discarded (Step 7), background fluorescence subtracted (Step 8, 9 and 10). These results are summarized in Figure 15. Finally, GBA activity (μg 4-MU/hr/mg total protein) average values and standard deviations were calculated, where activity in absence of chaperone treatment was set to 100% and results plotted as a histogram (Steps 11, 12 and 13). Final result of the measurement is presented in Figure 16.

Panel 1:

St. Curve [BSA] 10mg/ml 595nm	
0	0,001
0,2	0,026
0,4	0,043
0,6	0,049
0,8	0,073
1	0,081

Panel 2:



Panel 3:

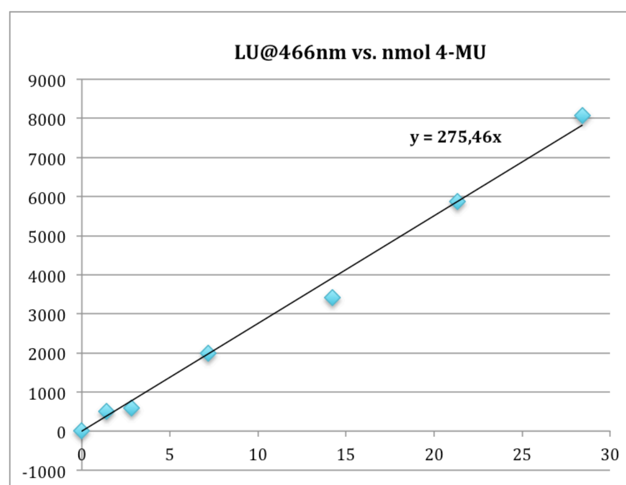
GD1 Chap.#1-#8	Sample Concentration µM	Abs. @ 595nm	Protein concentration (µg/ml)
1	5	0,061	0,702
1	30	0,073	0,84
1	50	0,083	0,955
2	5	0,075	0,863
2	30	0,065	0,748
2	50	0,085	0,978
3	5	0,051	0,587
3	30	0,069	0,794
3	50	0,051	0,587
4	5	0,057	0,656
4	30	0,054	0,621
4	50	0,07	0,806
5	5	0,074	0,852
5	30	0,055	0,633
5	50	0,074	0,852
6	5	0,055	0,633
6	30	0,043	0,495
6	50	0,079	0,909
7	5	0,068	0,783
7	30	0,074	0,852
7	50	0,043	0,495
8	5	0,062	0,713
8	30	0,063	0,725
8	50	0,08	0,921

Figure 14 - Bradford Assay on GD1 fibroblasts treated with different chaperones. Panel 1: Table showing concentration of BSA used [BSA](µg/µl) and the corresponding mean 595nm absorbance values for each dilution; Panel 2: resulting standard curve; four different BSA concentrations were used to determine the standard curve, with technical triplicates were performed for each point. Panel 3: Using the values of absorbance at 595nm obtained and the Bradford standard curve, protein concentration was calculated in µg/µl (taking into account dilution) for each sample.

Panel 1:

nmol 4MU	Adjusted Mean
0	0
1,42	510
2,84	585
7,19	1995
14,2	3425
21,3	5879
28,4	8068

Panel 2:



Panel 3:

Chaperone	Concentration µM	Abs.@466nm in triplicate		
none	0	585	590	356
#1	5	1568	1739	1544
#1	30	2876	2228	1520
#1	50	3074	2155	1836
#2	5	1279	1079	1207
#2	30	1483	1440	1330
#2	50	1594	1271	1456
#3	5	1246	676	795
#3	30	967	955	971
#3	50	933	1116	1124
#4	5	616	545	640
#4	30	529	593	653
#4	50	851	480	571
#5	5	2383	1807	1955
#5	30	1668	1764	1686
#5	50	1407	1506	1406
#6	5	1666	1471	1271
#6	30	2335	2149	1862
#6	50	1971	1852	1881
#7	5	2090	1787	X
#7	30	2255	1909	1821
#7	50	2115	1906	2013
#8	5	1229	1048	1150
#8	30	1033	895	862
#8	50	1363	1329	1011

Figure 15 - GBA fluorometric assay of GD1 samples and GBA standard curve. Panel 1: GBA standard curve data measured in triplicate. Anomalous readings were eliminated and are represented by 'X'. Panel 2: Standard curve plot. Panel 3: GBA assay fluorescence data measure in triplicate (with background fluorescence already subtracted).

Panel: 1

nmol 4MU/hr/ug Protein			
Chaperone	Concentration µM	Mean	St. Devia.
none	0	100,0	26,2
#1	5	264,8	17,4
#1	30	352,0	108,1
#1	50	433,1	118,2
#2	5	167,1	14,2
#2	30	245,6	13,7
#2	50	337,5	38,0
#3	5	189,9	63,1
#3	30	164,6	1,4
#3	50	170,8	17,4
#4	5	130,5	10,7
#4	30	128,6	13,5
#4	50	176,2	53,7
#5	5	310,0	45,3
#5	30	275,4	8,2
#5	50	400,1	16,0
#6	5	283,5	38,1
#6	30	316,0	35,6
#6	50	614,0	20,0
#7	5	367,6	41,7
#7	30	329,7	37,9
#7	50	407,5	21,2
#8	5	235,6	18,7
#8	30	182,2	17,8
#8	50	186,8	29,4

Panel 2:

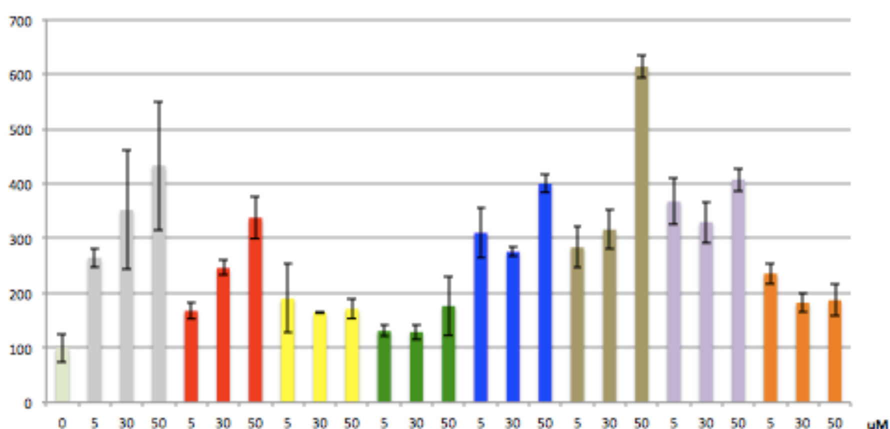
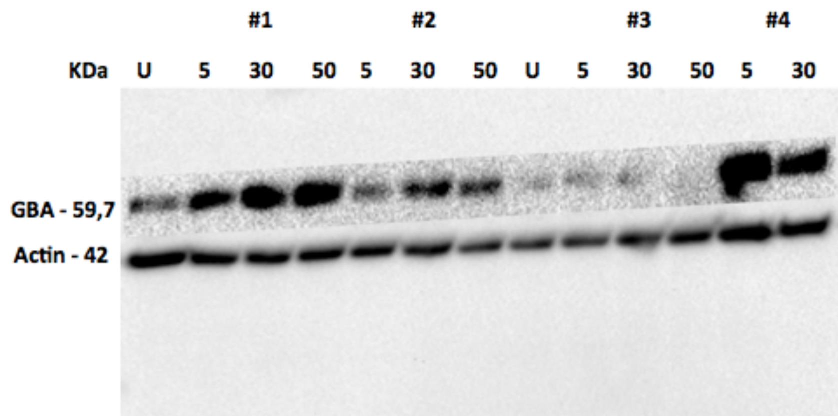


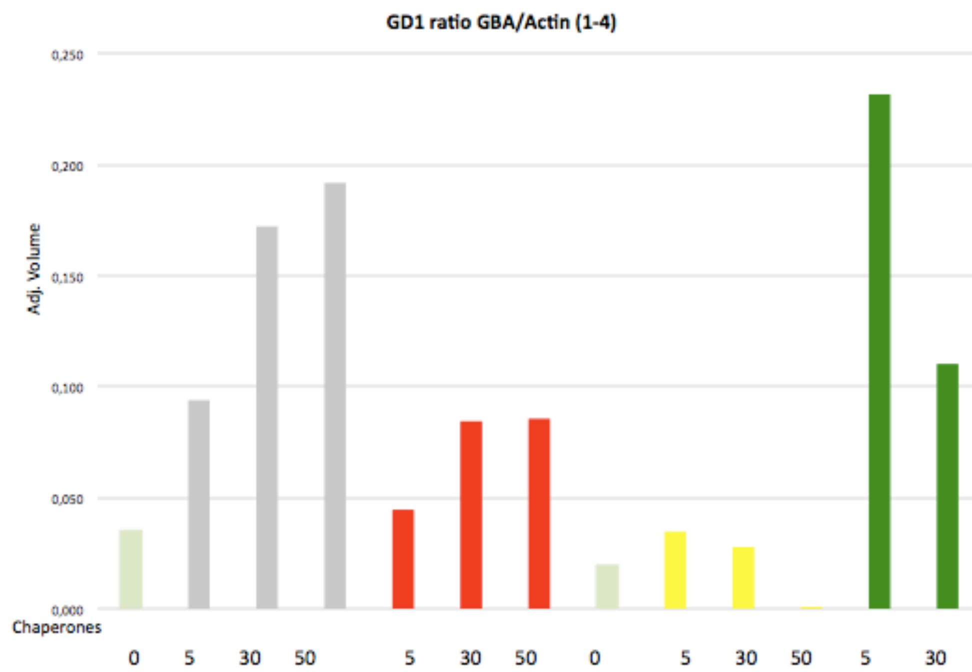
Figure 16 - Effect of chaperones 1-8 on glucocerebrosidase activity of fibroblasts (GD1) derived from a patient with GDT2 with genotype N188S/G193W. Panel 1: Table presenting average GBA assay values per treatment along with standard deviations (with GBA value for untreated cells set to an arbitrary value of 100%). Each colour is representative of the number of chaperone. Panel 2: Results presented as histogram chart.

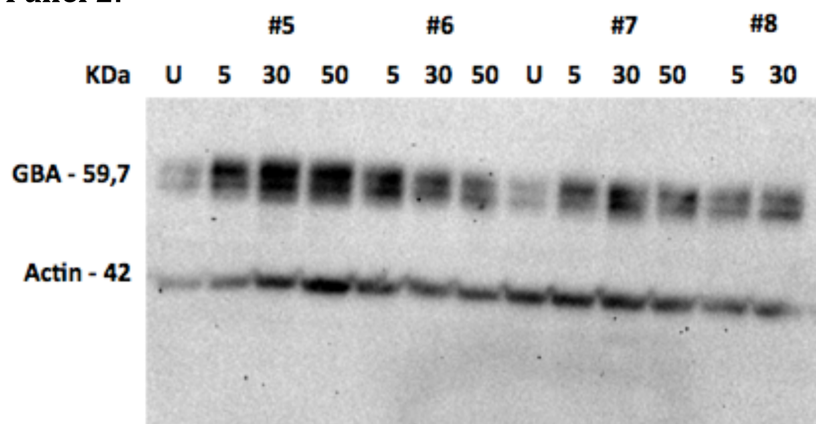
Overall, our results show that 7 out of 8 chaperones (excluding # 4) result in increased glucocerebrosidase activities. Increases range from 0,5 – 6 fold compared to glucocerebrosidase activity in untreated cells. For several of the chaperones, increases in GBA activity were proportional to the concentration of chaperone compound (#1, 2 and 5), while in others (#3, 5, 7 and 8) all concentrations tested seemed to increase GBA activity to similar levels. For these compounds, repeating the analysis with a lower range of concentrations might reveal the dosage sensitive concentration range. Compound #4 did not seem to increase activity, except at 50μM concentration, where increase was approximately 0,6 fold.

We then wondered whether the increase in levels of GBA activity revealed by the fluorometric assay would be reflected in protein levels as measure by Western blot. We therefore ran our protein extract samples (30 μg/lane) on a polyacrylamide gel, transferred the resolved proteins onto a PVDF membrane and probed it with antibodies against GBA and against Actin, as described in Materials and Methods. Results are presented in Figure 17.

Panel 1:







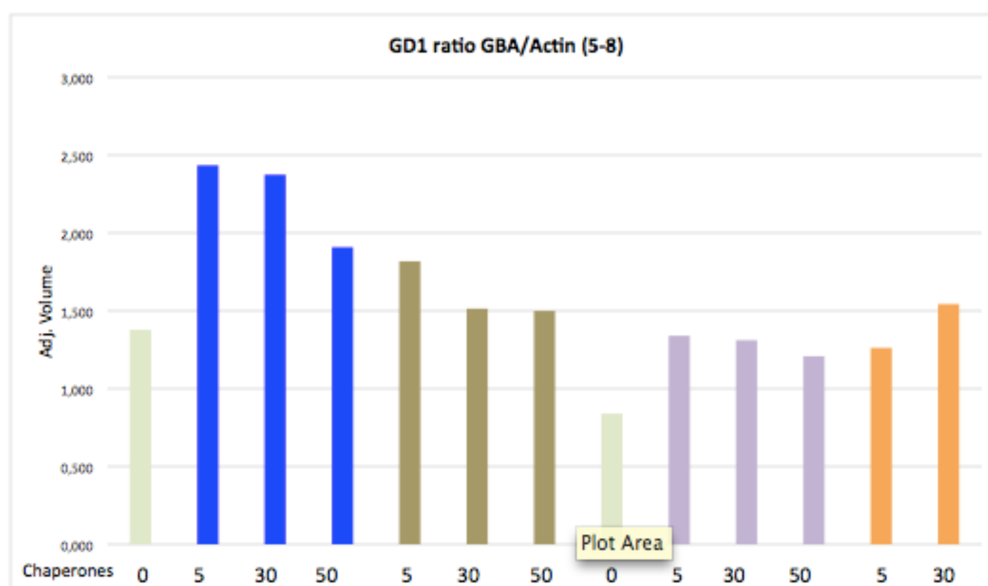


Figure 17 - Western Blot analysis of GD1 fibroblasts treated with increasing concentrations (0, 5, 30 and 50μM) of chaperones 1-8. Panel 1: Effect of chaperones # 1-4. Panel 2: Effect of chaperones # 5-8. Experimental details are described in Materials and Methods.

Based on the GD1 GBA activity assay we performed a Western Blot to confirm protein levels, as can be seen in Figure 17. We then obtained levels of protein expression by fluorescence quantification as seen on histograms in Figure 17 panel 1 and panel 2, showing the adjusted volume of GD1 samples.

Chaperone #1 has an intense protein expression as Western Blot and histogram shows, according to GD1 GBA activity assay. Chaperone #2 presents a certain protein expression, in 30μM and 50μM concentrations has a slight additional expression as seen in Western Blot and histogram, such as in G1 GBA activity assay. The protein expression of Chaperone #3 seems to be lower, almost unobservable unlike Chaperone #4, which presents a high expression in 5μM concentration and somewhat in 30μM concentration. Chaperone #3 and #4 are more or less according to GD1 GBA activity assay, might had more protein quantity on the gel. Particularly, 50μM concentration of Chaperone #4 may have been lost on the gel or in transfer to the membrane.

The strong protein expression observed in Chaperone #5 Western Blot and histogram is more less as shown in GD1 GBA activity assay. Chaperone #6 and #7 present some protein expression in Western Blot and histogram, according to GD1 GBA activity assay. Except in Chaperone #6 50μM concentration, which shows an increasing activity in GD1 GBA activity assay and in Western Blot there is not so much protein expression. As in Chaperone #7 protein activity seems to be higher than protein expression in Western Blot and histogram. There is some protein expression of Chaperone #8 in Western Blot and histogram, being high in 30μM concentration, comparing to GD1 GBA activity assay. Once again, Chaperone #8 50μM concentration in Western Blot seems to be lost in gel or in transfer. In general, GD1 samples protein expression shown in Western Blot is in conformity with protein levels of GD1 GBA activity assay.

In the case of GD1 fibroblasts (GDT2 genotype N188S/G193W) treated with 8 chaperone compounds, the results we obtained on the GBA assay were confirmed by Western Blot, suggesting that the GBA assay was accurate. Therefore, for GD2, GD3 and GD4 fibroblasts we performed the GBA assays but did not confirm them by Western Blot. In the following section we present the final results of the GBA fluorometric assay for GD2, GD3 and GD4 fibroblasts in Figure 18, Figure 19 and Figure 20.

3.3.2 Effect of 8 chaperone compounds on GD2 fibroblasts (GDT2 genotype F213I/RecNaI):

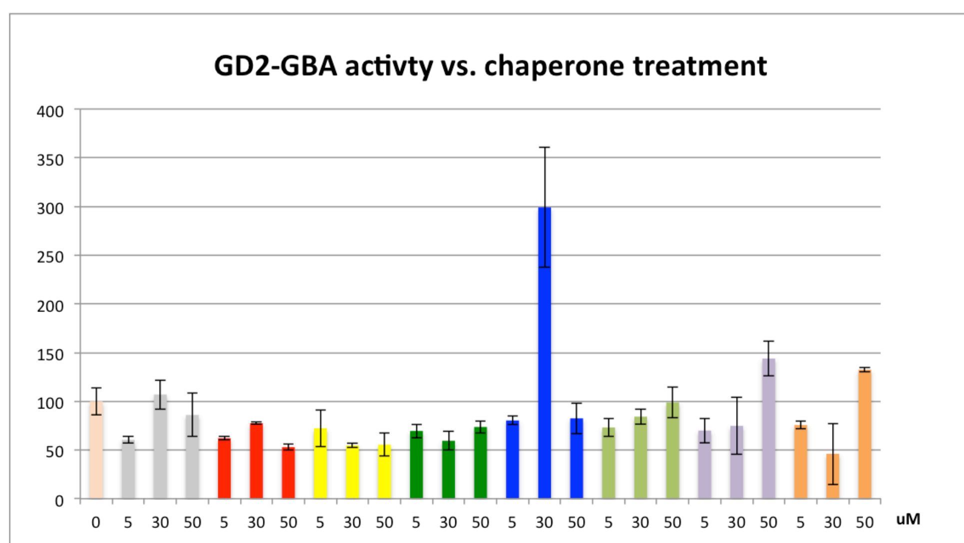


Figure 18 - Effect of chaperone 1-8 on glucocerebrosidase activity of fibroblasts (GD2) derived from a patient with GDT2 with genotype F213I/RecNaI. Protein activity of GD2 fibroblasts treated with 8 different chaperones in different concentrations (5μM, 30μM, 50μM) compared to protein activity of GD2 fibroblast control untreated sample (0). Results presented as histogram chart.

3.3.3 Effect of 8 chaperone compounds on GD3 fibroblasts (GDT2 genotype L444P/L444P):

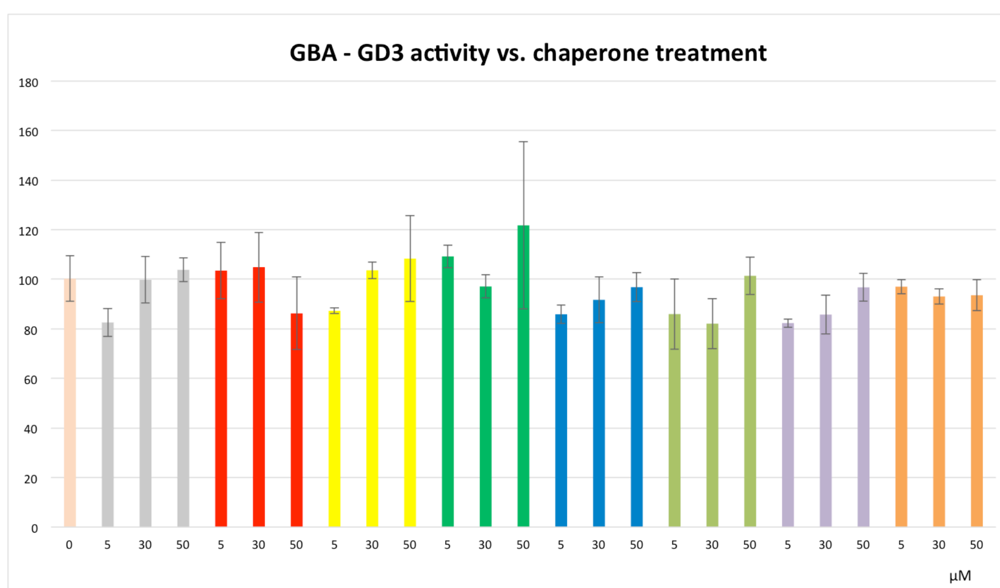


Figure 19 - Effect of chaperone 1-8 on glucocerebrosidase activity of fibroblasts (GD3) derived from a patient with GDT2 with genotype L444P/L444P. Protein activity of GD3 fibroblasts treated with 8 different chaperones in different concentrations (5µM, 30µM, 50µM) compared to protein activity of GD3 fibroblast control untreated sample (0). Results presented as histogram chart.

3.3.4 Effect of 8 chaperone compounds on GD4 fibroblasts (GDT2 genotype L444P/R120W):

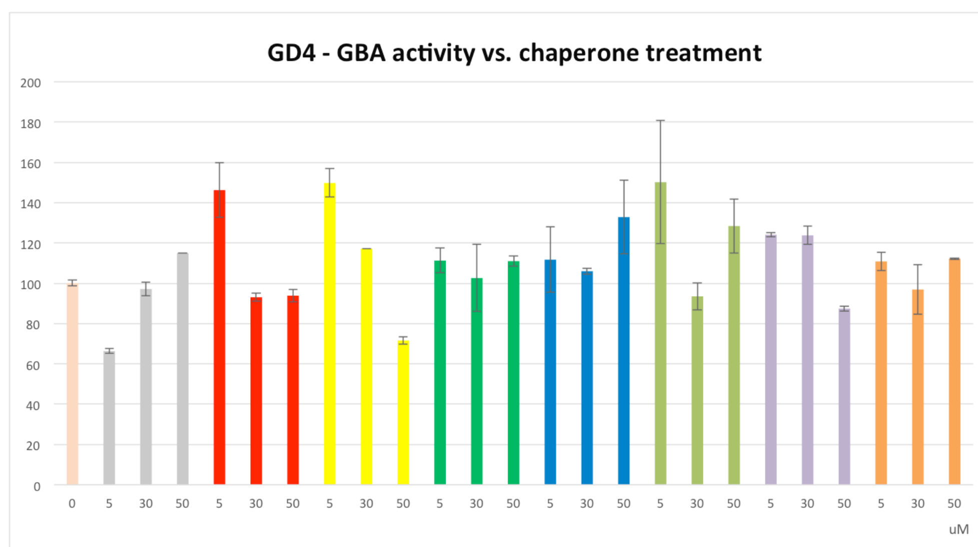


Figure 20 - Effect of chaperone 1-8 on glucocerebrosidase activity of fibroblasts (GD4) derived from a patient with GDT2 with genotype L444P/R120W. Protein activity of GD4 fibroblasts treated with 8 different chaperones in different concentrations (5μM, 30μM, 50μM) compared to protein activity of GD4 fibroblast control untreated sample (0). Results presented as histogram chart.

Overall, our results indicate that several of the chaperone compounds tested increase protein activity in the GD1 fibroblasts used in this study. Interestingly, this panel of chaperone compounds does not significantly improve protein activity in the GD2, GD3 and GD4 fibroblast lines tested, regardless of the concentration used. Compared to untreated cells, no increase in glucocerebrosidase activity was observed. Chaperone compound #5 seemed to increase GBA activity in GD2 fibroblasts at 30 μM by 3 fold. The fact that this

increase was not observed at 5 μM or 50 μM makes this measurement doubtful. Indeed, a closer examination of the protein concentration reveals that this particular sample seemed to have a protein concentration that was significantly lower than for all other samples in the experiment, explaining why the GBA activity is so high when normalized to protein content. We suspect that the protein concentration value was incorrectly measured, and that overall, this chaperone has no effect on GBA activity of GD2 fibroblasts.

Nevertheless, the effect of chaperone compounds in GD1 fibroblasts was in line with expectations. For several chaperone compounds an increase of GBA activity and stability was observed. In several cases, the increase was proportional to the concentration of chaperone. Our experiments would need to be repeated to confirm these results, and in that case, we would be able to confirm that the effect of a particular chaperone on glucocerebrosidase is dependent on the particular genotype. Possibly, different mutations affect the 3D structure of the enzyme in different ways and different chaperones may therefore have different effects. In addition, the capability of glucocerebrosidase-chaperone complex to be recognized as folded and transit through the ER depends on other constituents of each individuals' ER quality control machinery (Bendikov-Bar, Maor, Filocamo, & Horowitz, 2013). Consequently these facts may be the source differences in chaperone compounds effects on glucocerebrosidase activity of the samples used.

Chapter 4

Conclusion

As it is known, GD presents both visceral and neuronopathic symptoms, thus, therapies that address both types of symptoms are needed. The use of chaperone compounds in this work was mainly with the aim to contribute to overcome certain difficulties in GD therapies for the neuronopathic symptoms. Four different fibroblasts lines from GD patients with different genotypes of GDT2 were used in this study. These samples were tested with 8 different chaperone compounds to measure their glucocerebrosidase activity. According to our results, this method seems to be a promising approach in GD.

The fibroblasts used were from GDT2 patients with four different genotypes. Our results suggest that chaperones might be mutation specific in their effects, due to the differences found in results from patient sample GD1 and from results in patient samples GD2-GD4. Bendikov-Bar et al. proposes that the ability of the protein-chaperone complex to be recognized as folded and leave the ER is depending on components of the individuals' ER function. Furthermore, the increase in GBA activity observed for several chaperones in the case of GD1 suggests that some of these compounds could be used in a potential therapy, as clinical data suggest that a modest increase in glucocerebrosidase activity may be enough to achieve a therapeutic effect (Sawkar et al., 2002; Yu et al., 2007). In addition, chaperones remove mutant protein molecules from ER and increase lysosomal protein activity. As well as reduce significantly the ER stress load caused by accumulation of misfolded protein molecules, therefore chaperones protect cells from the consequences of ER stress (Bendikov-Bar et al., 2013).

The research in this therapeutic approach must continued, once the response to chaperones seems to depend on patients mutations. Also, the identification of more potential chaperone compounds for mutant enzyme variants is a goal in developing this therapy (Bendikov-Bar et al., 2013).

Chapter 5

Future Perspectives

In the near future, this approach and its techniques will be useful to test the effect of pharmacological chaperones in neurons derived from IPSc of GD patients. The goal is to determine its potential use for a GD therapy. These chaperones may be tested in neurons and can be a progress for neurological manifestations treatment in GD. Therefore this experimental work may contribute on the development of a prospective therapy for neuropathic forms of the disease.

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Annexes

RIPA reagent recipes:

RIPA Base stock

790mg TRIS-Base;

900mg NaCl;

Dissolve in 80ml;

pH 7.4 (with HCL)

Add 10ml of 10% Triton;

Add 2.5ml of 10% Na-deoxycholate solution;

Top up to 100ml.

Active RIPA Buffer

To make active RIPA buffer;

To 20ml of RIPA stock, add the following;

200ul of 100mM EDTA stock;

20ul of 1000x P.I.C. solution;

200ul of Na_3VO_4 (sodium orthovanidate);

1ml NaF (200mM stock);

Once this RIPA is made it is good for about 2 months.

Bradford reagent recipe:

Preparation of Bradford working dilution: Bradford Assay dye (BioRad # 500-0006) Dilute stock 1/5 in H_2O, filter, store at 4ºC, 2 weeks).

GBA Assay reagent recipes:

Glycine buffer:

Prepare 100ml of Glycine buffer: dissolve 1.5g of glycine, 1.32g of sodium carbonate, 0.64g of NaOH in 70ml mQ H20, adjust to 100ml final volume with mQ H20;

Substrate solution:

Prepare 100ml of Substrate solution: 0.169g of 4 MU-B-D glucosylpiranoside mw: 338.3 g/mol, 100ul of Triton X, 0.3g of Taurocholate, in Phosphate/Citrate buffer ph 5.8 (adapt values, calculate the amount you need in X g of 4MUBD);

4 methylumbelliferyl (4MU):

Prepare a 142mM solution of 4 methylumbelliferyl (4MU) in absolute ethanol: weigh 24.9 mg of 4MU and dissolve in 1ml of EtOH, dilute 1/100 + 1/100 + 1/10 (using ethanol), this will give you a solution of 1.42 nmol/ul.

Western Blot buffers recipes:

Running Buffer 10x

10g SDS
30,3g Tris
144,1g Glycine

800ml H2O

Up to 1L once dissolved.

Transfer Buffer 1X

2,9g Glycine
5,8g Tris
0,37g SDS

200ml Methanol

Up to 1L (H2O).

Western Blot Antibodies:

AntiGBA
AB4 - Mouse monoclonal to GBA 1x100 ug; abcam®discovermore; Ref.: 798203747835; Product: ab55080; Dilution: 6ul AB in 6ml BSA blocking solution.

AB6 - Goat anti-mouse IgG peroxidase-conjugated 0,8 mg/ml; Affinipure®; 115-035-003; Dilution: 1ul AB in 10ml BSA blocking solution.

AntiActin
Actin (I-19): sc-1616 - Actin goat polyclonal; Santa Cruz Biotechnology, Inc®; Molecular Weigh: 43 kDA; starting dilution 1:200, dilution range 1:100 - 1:1000; diluted in BSA blocking solution.

Goat anti mouse IgG-HRP: sc-2005; Santa Cruz Biotechnology, Inc ®; dilution: 1:5000.

Western Blot ECL: Amersham ECL Prime Western Blotting Detection Reagent; Detection reagent 1 and detection reagent 2; code number: RPN 2232; Dilute 1:2 PBS.

Western Blot Revelation: ChemiDoc (BIORAD®); Software: Image Lab 5.1 – Beta.