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Gaucher Disease: A Glance from a Medicinal Chemistry Perspective

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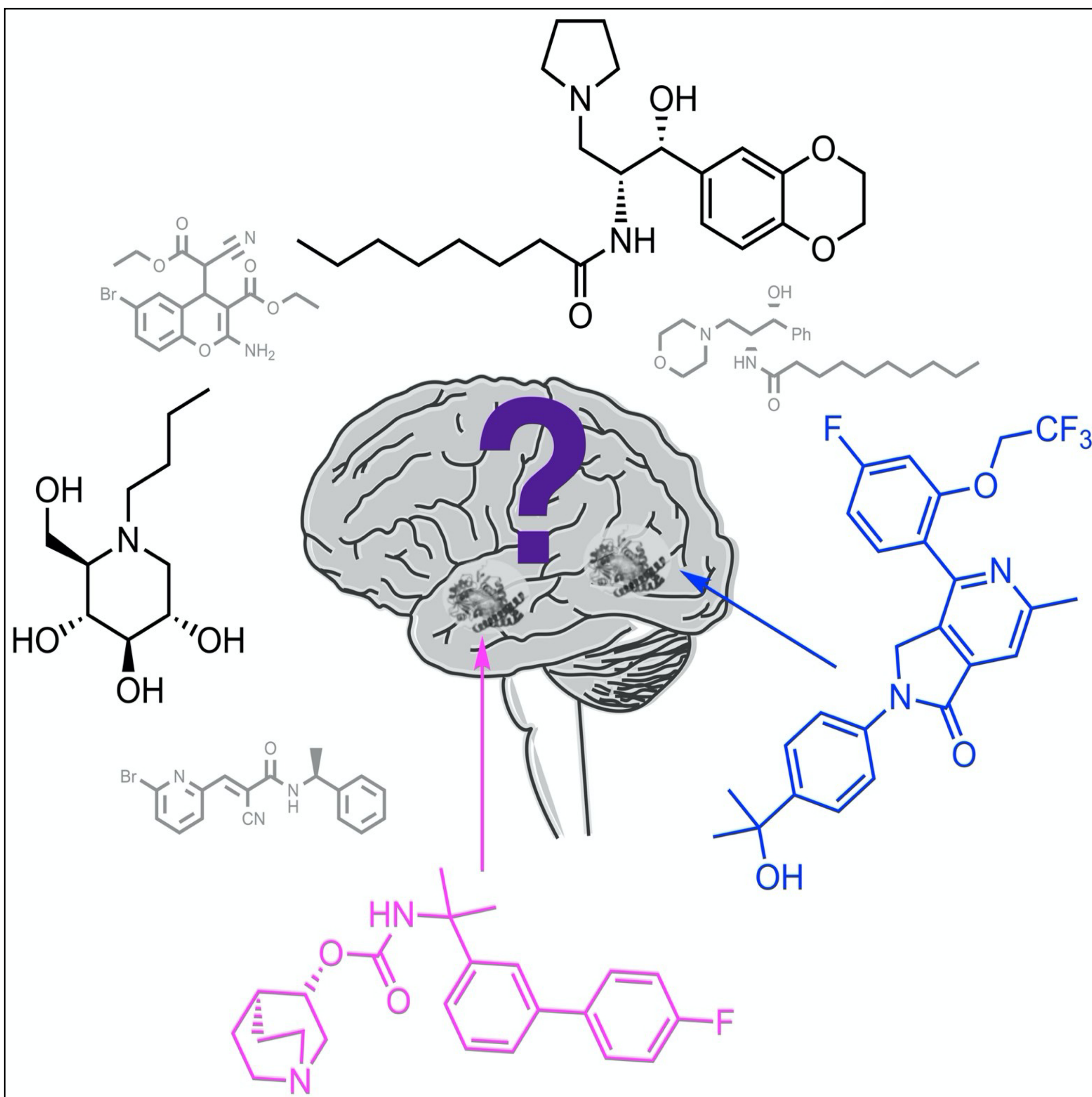

Dedicated to Prof. Maurizio Prato on the occasion of his 70th birthday

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Abstract: Rare diseases are particular pathological conditions affecting a limited number of people and few drugs are known to be effective as therapeutic treatment. Gaucher disease, caused by a deficiency of the lysosomal enzyme glucocerebrosidase, belongs to this class of disorders, and it is considered the most common among the Lysosomal Storage Diseases. The two main therapeutic approaches are the Enzyme Replacement Therapy (ERT) and the Substrate Reduction Therapy (SRT). ERT, consisting in replacing the defective enzyme by administering a recombinant enzyme, is effective in alleviating the visceral symptoms, hallmarks of the most common subtype of the disease whereas it has no effects when symptoms involve CNS, since the recombinant protein is unable to significantly cross the Blood Brain Barrier. The SRT strategy involves inhibiting glucosylceramide synthase (GCS), the enzyme responsible for the production of the associated storage molecule. The rational design of new inhibitors of GCS has been hampered by the lack of either the crystal structure of the enzyme or an in-silico model of the active site which could provide important information regarding the interactions of potential inhibitors with the target, but, despite this, interesting results have been obtained and are herein reviewed.

1. Introduction

A disease is considered rare if it affects only a very limited number of people in the total population. The exact definition of rarity is different in Europe than in the US: while in the Old Continent a prevalence of about 5/10000 births is required for a disease to be considered rare, in the US this definition applies when less than 200000 people are affected. In epidemiological studies, parameters such as incidence and prevalence are used: incidence refers to the number of people who develop a particular disease or experience a particular health-related event in a given time period, while prevalence, the most commonly used parameter, refers to the total number of people in a population who have a disease or health condition at a given time, and is usually expressed as a percentage of the population. A comprehensive study by N. Wakap *et al.*,[1] based on data from the "Orphanet Epidemiological file"[2] and excluding rare tumors, rare infectious diseases, and poisonings, found rare diseases prevalence of about 3.5-5.9% of the world population, *i.e.* 263-446 million people. However, there are no universally accepted standard parameters that would allow a clearer and more unambiguous definition of rare diseases.[3]

These diseases are usually genetic and lead to severe disabilities, so that most of those affected die before the age of five. There are few specific therapeutic approaches to successfully treat rare diseases, and only a few hundred approved compounds are currently available on the market. In general, correct diagnosis is very complicated and rarely immediate; drugs, when available, are usually expensive, and there are no uniform, accepted guidelines for the patients' treatments. In addition, genetic variability makes it difficult to evaluate pharmacological protocols, which are at the same time strongly influenced by external factors such as diet, lifestyle, and environment. Although the search for new treatment options has increased in recent years, there are still rather few studies and protocols, usually based on feedback from patients, actively involved in shaping drug therapies.[4]

The so-called orphan drugs are molecules that are essential for the rare diseases therapy. According to Orphanet, a global network that provides scientific data and expertise of professionals on rare diseases, orphan drugs are "medicines that are not developed by the pharmaceutical industry for economic reasons but that respond to a public health need". Moreover, a drug may also be considered as "orphan" when a substance may be used in the treatment of a frequent disease but may not have been developed for another, more rare therapeutic indication.[5-7] Indeed, pharmaceutical companies have always shown little interest in these types of therapies. However, this trend is changing, in the US thanks to the "Orphan Drug Act" and other financial incentives for companies investing in this field, and in Europe thanks to some tax breaks and the attention given to this issue by the new Research Executive Agency calls under the Horizon Europe programme.[8-10]

According to the EU definition, orphan drugs must meet three main criteria: i) they must be specifically indicated for the treatment of a life-threatening or chronically debilitating disease; ii) they must be specifically indicated for the treatment of a particular rare clinical condition, based on the current EU definition; iii) they must represent a significant clinical improvement over existing treatments, where such treatments are available.[11,12]

The drug development process for a rare disease raises more issues than that of a "classical" drug, such as the difficulties in distinguishing between different phenotypes of the disease or its evolution during the clinical treatment. In addition, the standard method of randomizing patients and treatment with both the drug and the placebo in clinical trials is not always possible due to the limited number of potential participants to the study. This, along with the geographical dispersion of both patients and researchers, affects the feasibility of the studies, leading to a high variability.

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To encourage medical research in the field of rare diseases, the International Rare Diseases Research Consortium (IRDiRC) has established specific guidelines to be followed in such studies:
i. the research methods used must be proportionate to the clinical conditions;
ii. safety data must be adequate and as comprehensive as possible;
iii. different project designs may be used;
iv. decisional and rational analytical approaches may be combined to adjust the level of evidence in clinical practice;
v. it is possible to extrapolate data from pharmacokinetic and pharmacodynamic models, off-label studies and patient records;
vi. patient feedback could be taken into account in the design of the clinical trial.[13]

Although representative patient populations are not always available for orphan drugs, post-approval studies are essential to a better understanding of the drug and its effect on patients, especially in pediatrics and in patients with complex clinical symptoms, which in turn result not only from an imperfect correlation between genotype and phenotype, but also from lifestyle together with concurrent pathologies and already in progress additional therapies.[14]

In addition, in 1983, a special law, the aforementioned Orphan Drug Act, was passed in the USA to facilitate the development of orphan drugs. It provides for tax breaks for pharmaceutical companies investing in this area of research, seven years of market exclusivity and simplification of the approval procedures, especially with regard to the clinical data required for approval. As a result of this law, the number of medicines approved for the treatment of rare diseases increased from 38 before 1983 to 373 in 2014. Later on, the EU introduced Regulation (EC) 141/2000[11] and Regulation (EC) 847/2000,[15] which established the criteria and protocols for the development of orphan drugs, the financial incentives associated with this status and the authorization procedures through the European Medicines Agency's (EMA) "Committee for Orphan Medicinal Products" (COMP).[16]

Moreover, the profitability of orphan drugs could be higher than expected, as companies producing generic drugs are often discouraged from entering this specific market due to the limited consumer base of these molecules. On the other hand, the off-label use market segment of an orphan drug could be tapped by the pharmaceutical company if that drug can be used for other therapies, leading to increased use of the drug and consequently higher revenues.[17,18]

2. Lysosomal Storage Disorders

Lysosomes formation is a very complex process deriving from the fusion of vesicles that have budded off from the trans-Golgi, and from the plasma membrane, releasing their contents, fluid and molecules from the extracellular environment, in the lumen of lysosomes, where they are processed.[19] There are about sixty different hydrolytic enzymes in lysosomes, which can be classified according to the type of molecules they hydrolyze, such as nucleases, phosphatases, lipases, and sulfatases. The mRNA for each of these hydrolytic enzymes is translated on polysomes bound to the endoplasmic reticulum (ER) membrane, then the newly assembled protein is transferred to the lumen of ER, where both the removal of the "signal peptide" and the glycosylation of the asparagine residues take place.[20] The enzyme is then transferred from the ER to the Golgi apparatus, where it is further functionalized with mannose-6-phosphate units, and finally, thanks to vesicular trafficking, it is transported to the lysosomes, and internalized by specific receptors, after recognition of molecular elements, such as mannose-6-phosphate.[21] The lysosomal lumen is characterized by a constant acidic pH, which is necessary for the proper activation and functionality of the enzymes. In addition to the function of metabolic degradation, lysosomes also play an important role in processes related to the immune system and the response to nutrients that contribute to cellular homeostasis. Given the relevance of lysosomes in the process of cellular signaling, it is quite clear that diseases affecting the functionality of lysosomes also involve other organelles related to their function.[22]

Lysosomes have a role in many diseases such as lupus erythematosus, neurodegenerative diseases, neurological autoimmune diseases, and rheumatoid arthritis. Among them, however, are also the so-called Lysosomal Storage Diseases (LSD), that are characterized by impaired function of enzymes in the lysosomal lumen, resulting in an accumulation of enzyme substrate both in the lysosomal lumen and in the cell. There is a large number of enzymes that can cause LSD, and the clinical expression of the disease depends on a) the specific class to which the enzyme belongs, b) its substrate, and c) the cells and organs primarily affected by the substrate accumulation. Depending on the physiological role of the enzyme, severe symptoms may be observed simultaneously in different organs. Therapeutic options targeting lysosomes are limited, although, due to their complexity, several approaches have been proposed to target and alter pathological processes. These are based on the use of molecular chaperones, substrate reduction therapies, inhibitors of cathepsins, ATPase proton pumps or calcium ion channels, modulators of phosphatidylinositol kinase or farnesyltransferase. However, the major drawback of these approaches remains the transport and specific release of the drugs in the lysosomes.[23]

The clinical manifestations of LSDs are very heterogeneous. The age of onset and progression of the diseases may vary according to the specific metabolic disorder and are consequently related to the genetic mutation that triggers the disorder, to the functional impairment of the enzyme and to the amount of unmetabolized substrate that accumulates in different tissues. This latter aspect is strictly dependent on the percentage of residual metabolic activity of the enzyme compared to the physiological level of enzymatic activity in the respective tissue. In addition, the environment influences metabolic fluxes and contributes to the clinical manifestation of the disease, thus there is usually no strict correlation between genotype and phenotype in LSD.[24]

The unmetabolized substrates that accumulate are glycosphingolipids (GSL) (Fig. 1), amphiphilic lipid molecules consisting of a sphingosine linked by an amide bond to a long-chain fatty acid and a sugar. The acylation of sphingosine, catalyzed by the acyltransferase, leads to the formation of ceramide, which acts as the backbone of sphingolipids and as such is an important resident of the plasma membrane of eukaryotic cells.[25]

Glycosphingolipids are mainly found in the cell membrane and are involved in signaling functions related to cellular proliferation, differentiation, and apoptosis. The hydroxy group at position 1 of the sphingosine core can be linked to several polar substituents that make the resulting molecule amphiphilic, leading to the

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production of glycosphingolipids, cerebrosides, sphingomyelin and gangliosides.

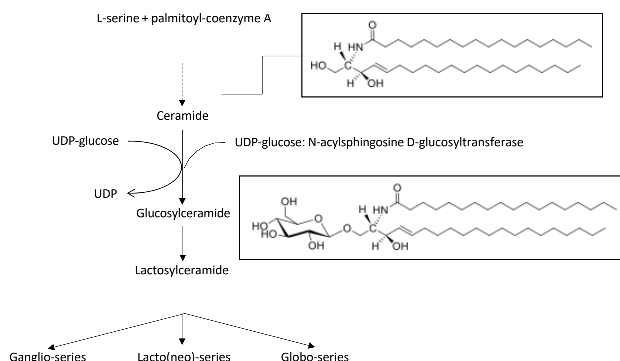

Figure 1. Example of a synthetic path for glicosphingolipids.[26]

Glycosphingolipids are characterized by the presence of a polar glucidic head, which is introduced by glycosyltransferases. The reaction is catalyzed by glucosylceramide synthase (GCS) and leads to the synthesis of glucosylceramides, while galactosyltransferase catalyzes the synthesis of galactosylceramides. Starting from these primary base molecules, various more complex derivatives, such as gangliosides and globosides, can be obtained by introducing different glucidic moieties.[27] Glycosphingolipids are synthesized in both the ER and the Golgi apparatus and are abundant in eukaryotic cell membranes. They are often found on the outer leaflet of the cytoplasmic membrane, internalized during endocytosis and then metabolized in lysosomes. The internalization of both endogenous and exogenous molecules into lysosomes is promoted by various mechanisms such as phagocytosis, pinocytosis, endocytosis, autophagocytosis, and the transport of certain proteins mediated by chaperones. In addition, specific carriers within the lysosomal membrane promote the recovery of molecules metabolized in the cytoplasm by internalizing them into the lysosomal lumen.[24] While glycosphingolipids were previouslythought to function exclusively as membrane components, they have now been found to be involved in several basic physiological metabolic processes and their imbalance can lead to various pathological conditions.[28]

LSDs are inherited autosomal recessive metabolic diseases, so that in heterozygous individuals the presence of the mutated gene on the chromosomes does not lead to the onset of the pathology. In some cases, they can be caused by a group of genes mutations, while in others a single crucial mutation is sufficient to cause the disease. However, it has been demonstrated that the full spectrum of symptoms associated with the pathology results from the combination of genotype, phenotype, and environmental factors, which in turn influence the residual activity of the enzymeand thus the onset of the disorder.

Lysosomal disorders can affect not only specific enzymes but also receptors, transporters, and effector proteins inducing the accumulation of substrates implied in the physiological processes that they control (Fig. 2). Oligosaccharidosis, gangliosidosis, sphingolipidosis, mucopolysaccharidosis are LSDs which lead to the accumulation of different metabolites, inducing a functional impairment of the cell which gradually spans several tissues and organs. The most affected organs are the liver and spleen due to the high expression level of the unpaired enzymes responsible for the disorder and because of their involvement in relevant and very active metabolic pathways.

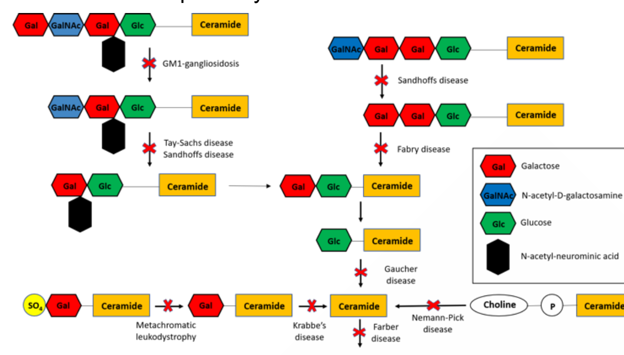

Figure 2. Schematic representation of various LSDs.[29]

The age of onset of such diseases is variable as well as the severity of the symptoms, the organs affected, and the prognosis. Also, the symptoms affecting the central nervous system (CNS), when present, can be very fickle even among individuals affected by the same disorder and belonging to the same family.[30] Frequently, the onset of the neurodegenerative disorder in infants is not imputable to a single LSD but rather to a series of LSDs.[28] The resulting pathological phenotype is severe when the impaired protein is completely inactive to block the related metabolic pathway. If instead the enzyme retains some residual functional activity, the symptoms are usually milder.

3. Gaucher Disease

3.1. Background Knowledge

In 1882 Philippe Gaucher described for the first time a particular pathological condition, which was named after him, the Gaucher disease (GD). The pathology remained completely misunderstood until 1901, when Brill found out that it was hereditary.[31] In the following years new knowledges were acquired, as the discovery of the toxicity at CNS.[32] The prevalence of the neurological forms of the disease during the childhood was noted, probably due to its early lethality which prevents individuals carrying the pathology to reach adulthood. In 1965 Brady *et al.* were able to analyze in detail the physio-pathological effects of the GD,[33] and later the mutated gene responsible of its onset was successfully cloned.[34] GD was then included within the "rare diseases" due to its low incidence in the population.[29]

This condition belongs to the group of diseases caused by LSDs within the sub-group of sphingolipidoses (Fig.3). The symptoms and the age of onset are variable but usually an early diagnosis results in a severe symptomology and, despite the occurrence of the same genetic mutations, clinical manifestations can range from almost unnoticeable to the involvement of vital organs and severe disabilities. The incidence of the pathology in the population is not constant, there is indeed a predisposition of certain individuals, such as the Ashkenazi Jewish community.[29]

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Three clinical forms of the disease have been identified, based on physiological and, more importantly, pathological features displayed by affected individuals, the most common of which is the Type 1, accounting for the 95% of the total cases. This classification is very useful to predict the prognosis and to undertake a specific treatment avoiding either unpleasant acute or irreversible episodes. GD Type 1 (GD1) is also less lethal because of the lack of cerebral symptoms, which to date cannot still be satisfactorily treated.

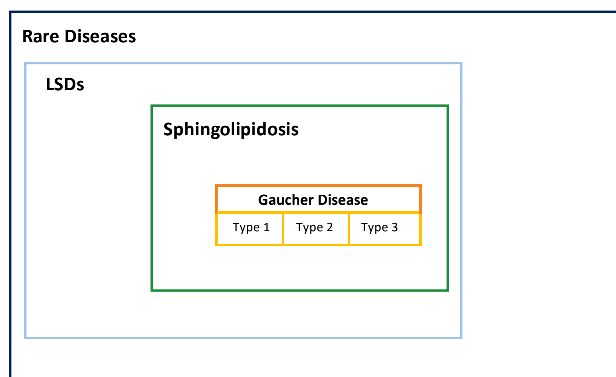

Figure 3. Relation of GD with respect to Rare Diseases and its subtypes.

GD1 usually takes a slow course, beginning with hematic abnormalities and ending up with involvement of several organs.[35] The most common symptoms involve peripheral organs, in particular bone, spleen, and liver. The increased amounts of released proinflammatory cytokines due to macrophages hyperactivation lead to several type of abnormalities. The musculoskeletal symptomatology, although not the most typic of GD, is highly disabling and common (70% of cases).

Among the clinical forms which display an involvement of the CNS, the GD Type 2 (GD2) is particularly lethal and is also known as "acute" or "juvenile", due to a poor prognosis within the second year of age. The GD Type 3 (GD3) is characterized by neuronal symptoms, but milder compared to GD2, and in addition it shows a delayed onset and a better prognosis.[36] The symptoms are similar to those of the GD1, with the addition of the neuropathic component.

GD has an autosomal recessive inheritance and equally affects male and female. The mutated gene, GBA1, is located on chromosome 1 and encodes for the enzyme Glucocerebrosidase (GCase), which is involved in the lysosomal lipidic catabolism. The mutated GCase becomes unable to hydrolyze glucocerebrosides or glucosylceramides (GlcCer) into ceramide and glucose.[37] The functionality of the enzyme can be partially or completely lost thus resulting, respectively, in the partial or null hydrolysis of glycosphingolipids within the macrophages, which represent the cell line mostly involved in the metabolism of cellular components. As already explained, GCase disfunction is responsible for the accumulation of its substrate within macrophages' lysosomes, inducing their transition into a hyper-activated state. This causes the production of pro-inflammatory mediators, as IL-1, IL-6, TNF-α, IL-10, M-CSF which worsen the disease symptoms.[37] These cells, known as Gaucher cells, grow up in size from 12 to 20-100 micron, tend to accumulate in specific tissues and organs, inducing several lesions and inflammatory conditions that add up to the hormonal and metabolic disfunctions caused by the disease.[29,35] Moreover, Gaucher cells have small eccentric nuclei and cytoplasm with a "crumpled tissue paper" appearance due to the presence of fibrillar aggregates. The organs mainly involved are spleen, liver, gastrointestinal apparatus, skeletal, blood, and bone marrow tissues but, in GD Type 2 and Type 3, the CNS, and the respiratory system are also involved.[37]

In general, some Gaucher-related symptoms may result from the presence of disrupted proteins within the same GCase metabolic pathway, as observed in a minority of patients with a defect in saposin C, (SapC) a non-enzymatic protein activator of sphingolipids that is critical for GCase functionality.[38,39] GD may also be caused by various defects in metabolic pathways involvedin the GCase production and its (lysosomal and not lysosomal) transport. If the enzyme is structurally unstable, it could lose its conformation before reaching the site of action and become subject to proteasome degradation.[40]

The lipids, accumulated in the lysosomes and in the cytoplasm, undergo several metabolic pathways, triggering the apoptosis process. The calcium homeostasis mechanisms are lost or impaired,[41] and the blood cells can be affected.[42] The abnormal presence of lipids leads to lysosomal impairments, with increased pH within the organelles and disruption of pathways between lysosomes and other cellular loci.[43] The cellular and extracellular signaling mechanisms are imbalanced, compromised mitochondria and misfolded glycoproteins tend to accumulate in the cytoplasm along with waste products at the ER level. The alteration of the membrane electric potential, generated by the electron transport chain, is one of the markers of mitochondrial damage, and it becomes lower along with the amount of oxygen consumed. This can be attributed to a diminished fluidity and plasticity of the Gaucher cells membranes, due to an impaired ratio between cerebrosides and GlcCer. Furthermore, the ceramide can also undergo self-assembly within the external mitochondrial membrane, forming stable channels enabling the release of pro-apoptotic molecules.[39]

3.2. Genetics and Correlation with Phenotype

It has been noted that patients sharing the same genotype display different symptomatology, and, at the same time, it is likely that individuals with different genetic background can display analogous symptoms. The manifestations that are not strictly related to the genotype make both the diagnosis and the prognosis very hard since there are not specific elements able to indicate the severity of the disease or its progression. A confirmation of this is the symptomatologic discrepancy observed in homozygous twins.[44]

The GD is inherited in an autosomal recessive pattern through the parental chromosomal transmission. Even though the disease is caused by mutation of a single gene, the related phenotypes are various and heterogeneous, ranging from asymptomatic individuals, whom can be diagnosed only through genotyping or by evaluation of the enzymatic activity, symptomatic individuals with mild symptoms up to severe problems which threaten patients' survival. More than 400 mutations of the GBA1 gene have been identified so far, only partially accounting for the great phenotypic heterogeneity as well as the prognostic differences.[45,46] In the USA, according to the National Gaucher

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Foundation, the estimated incidence of GD1 is 1/20000 live births, generally caused by N370S and 84GG mutations. In Turkey instead, the most common mutations are L483P and N409S, followed by D448H and L335V.[45]

The severity of the disease depends on the combination of the genomic mutations of each individual. There are some mutations that do not lead to severe symptoms, such as N370S, also called missense mutation, while others result in a complete loss of the protein functionality, such as the 84GG mutation, known as non-sense or frame shift mutation, which completely alter the reading frame of the codons.[47] The occurrence of the mutation N370S on one or both alleles of the GBA1 gene on the chromosome 1 indicates the presence of GD1. This is the most common mutation within the Jewish population with an incidence of 70-75% of mutated genes against a 23% incidence among non-Ashkenazi communities, it is very frequent, it does not lead to the onset of neurological symptoms, but it seems to prevent them. Several studies on the specific type of impairment of the Glucocerebrosidase due to the mutation N370S have been performed revealing that both the transcription and the translation of the enzyme take place correctly as well as the transport to the lysosome. The mutated protein shows the same affinity for the specific substrate but is not able to catalyze the hydrolysis of the glycosidic bond.[48] A heterozygous condition of this mutation generally results in a GD1 phenotype with mild, almost undetectable symptoms. In addition, there are L444P and D409H homozygous conditions which lead to the onset of CNS symptoms at the age of 2.3 years, at least for L444P.[35]

3.3. Diagnosis

The correct diagnosis of the Gaucher disease is, in most cases, established several years after the onset of the early clinical symptoms, due to the difficulties in correctly recognizing and establishing its symptomatology, given the limited experience in the field of rare diseases. Moreover, these pathologies are characterized by a progressive increase of both the severity of the symptoms and the number of involved sites.

The disease can be evaluated using several available methods, according to the complexity of the pathological manifestations and the availability of the analysis laboratory.

1. Evaluation of the residual activity of the Glucocerebrosidase: measured in leukocytes, in cultured fibroblasts or in amniotic cells.[38] In case of disease, the residual enzymatic activity is around 10-15% of the total expected. Although this test is the screening method of choice, it does not provide information about the severity or progression of the disease itself.[35,37,49] The flow cytometry of monocytes from the bloodstream could represent a more accurate method for evaluating the pathological condition, but it has not been yet validated and therefore is not officially approved.[50]
2. Bone marrow aspiration: can be performed in patients displaying thrombocytopenia and hepatosplenomegaly. It is very useful in the identification of Gaucher cells, but, even though the test is very reliable, if the sample does not contain an adequate number of cells, it can produce insignificant responses. Another limitation derives from the potential misreading of Gaucher cells and pseudo-Gaucher cells which can be observed in some infective-type disorders or in disorders caused by a high cellular turnover, such as myeloma, lymphoma and leukemia.[35,37,49] Together with all the pros and cons, it is useful to confirm thepresence/absence of hematological malignancy by hematic counting after enzyme replacement therapy.[45]
3. PCR evaluation of the GBA1 gene mutations, focused on the identification of the five most relevant mutations. A negative response does not rule out the presence of the disease because of the limited number of screened mutations, although they are the most common ones. This test is particularly employed enabling the family screening,[37,49] and there are molecular tests focused on a single gene or multiple genes, in order to perform a targeted analysis of the most common pathogenic variants.
4. Serum biomarkers: chitotriosidase (CT) concentration, ACE (Angiotensin Converting Enzyme), CCL18 (Chemokine(C-C motif) ligand 18), tartrate-resistant acid phosphatase and ferritin can be relevant for the diagnosis of GD.[49]
5. Algorithms of diagnosis: they have been set up based on studies accounting for hundreds of patients from ten different countries allowing to emphasize the peculiarity of the Gaucher disease, pointing out the differences related to the specific ethnic groups. Taken together, this information allowed to construct algorithms which facilitate a correct diagnosis, corroborated by specific exams for the identification or the exclusion of the pathology.[51]

The hepatosplenomegaly has a particular relevance for the diagnostic evaluation because it is strongly associated with the GD; however, it cannot be considered a unique indicator of the disease since it is observed also in other pathologies. Similarly, the analysis of the circulating red blood cells is not sufficient to uniquely discern GD from other LSDs. Moreover, to avoid a potential diagnostic error, it is important to identify the eventual deficiency of SapC (or pro-saposin OMIN 610539). There are patients with a mutation in the PSAP gene, encoding for the saposin, which is transmitted to the progeny in an autosomal recessive pattern. This leads to Gaucher-like pathological manifestations, both at peripheral and neurologic levels, with lipids accumulations in several sites of the organism, despite the normal enzymatic activity of the Glucocerebrosidase. Saposin, indeed, is a crucial co-factor of the GCase for the hydrolysis of the glycosphingolipids and it has been shown to be very useful for the protection of the GCase itself, preventing its degradation or its inhibition promoted by α-synuclein.

3.3.1 Score Index

Gaucher disease can be assessed by using the score index of clinical severity, based on the symptomatology displayed by the patient. The first score index was introduced by Zimran, the *Zimran Severity Score Index* (SSI).[52] Another classification is the *Gaucher Disease Severity Score Index Type I* (GauSS-I),[53] developed to obtain an extensive and reliable method to identify and to correlate genotypes and phenotypes displayed by patients, and to associate them with the biological and clinical markers which characterize the disease. With this approach, it is possible to identify the clinical variability and the severity of the disease and choose a tailored therapy. According to this methodology, there are six main aspects, presence of biomarkers, and effects on skeletal, hematological, visceral, pulmonary, and neurological systems, the severity of which is evaluated.

3.3.2 Markers

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The most used markers to evaluate GD are chitotriosidase and chemokine CCL18. The CT is an enzyme secreted by macrophages, whose role is to process chitin. It has an essential role of remodeling, especially in tissue healing and chemotaxis of the immune system cells. High levels of the protein are detected not only in the case of LSD, but also in other disorders such as sarcoidosis, thalassemia, leishmaniasis and leprosy.[36] Before dosing the level of the marker, it is pivotal to verify that the patient does not have a mutation in the gene encoding for the CT. In this case the CT cannot be considered a useful marker, but otherwise untreated GD is characterized by very high levels of this molecule, 100-5000 times higher than the physiological levels. Usually, the higher the levels the highest the severity of the disease, and their values tend to drastically decrease, along with the most evident symptoms, after an efficient treatment.[54]

As mentioned, there is a second, very specific marker *i.e.*, chemokine CCL18, secreted in the lungs, whose levels are 30 times higher in non-treated patients. CCL18 is a small molecule which is filtered by kidneys and its urinary concentration is proportional to the hematic concentration. The analysis of the protein is usually followed by a radiological check-up of the bone marrow and of the skeletal tissue.[36] Despite its specificity, CCL18 is not the marker of choice because of the high dosage complexity in the routine tests;[35] therefore it is used as marker only in those patients carrying the chitotriosidase gene mutations.

For the routine monitoring, several other markers can be employed such as tartrate-resistant acid phosphatase, ACE, hexosaminidase, cathepsin K, and also the levels of interleukins such as IL-1β, IL-6, IL-10, TNF-α, macrophages inflammatory proteins MIP-1α, MIP-1β and CD-163.[36]

Another important biomarker for the disease is the glucosylsphingosine (Lyso-GL1 or Lyso-Gb1), the deacetylated form of glucosylceramide, that increases if there is an accumulation of its precursor in the cells. The plasma levels in healthy state are around 1.5 ng/mL, while in the case of the disease reach 181 ng/mL.[55] Its concentrations can be determined also to monitor the follow up of the therapy, with a marked decrease of Lyso-Gb1 when the treatment is successful. In fact, in some cases, it was even possible to detect almost normal level of this marker in stable, properly treated GD1 patients.[56]

As recently reviewed,[57] increased levels of Lyso-Gb1 were also found in GCase-associated Parkinson's Disease and they are also related to the B-cell lymphoproliferative disorders as multiple myeloma, and, also in these cases, their concentration decrease is a positive sign.

Despite all these possibilities, there is still a constant search for novel specific biomarkers to improve the diagnosis.

4. Therapy

Considering the varieties of symptoms that can characterize this pathology, some patients do not need specific treatments, whereas for others it is more convenient to deal with the disease symptoms rather than with the side effects of the therapy. Patients with severe conditions, instead, need an extensive study of the phenotype for the choice of the most effective therapeutic approach.

Before 1991, the GD therapy was mainly focused on alleviating the symptoms as for the splenectomy in patients with severe spleen impairment. This approach usually led to rapid and evident improvements, with normalization of platelets count, diminution of fatigue and hemorrhages. Thereafter, several alternatives to surgery have been proposed, given the need for a therapy which would alleviate in a more effective way the severe symptoms, considering the impossibility to completely eradicate the disease. To date, splenectomy is only an emergency procedure that has been associated with relapse, worsening of the condition of the musculoskeletal system, hepatomegaly with the tendency to alter hepatic functionality, eventually resulting in cirrhosis, and a higher probability to develop septicemia.[35,58]

The most updated and important approaches to treat GD are the enzyme replacement therapy (ERT), and the substrate reduction therapy (SRT), but other pharmacological strategies are available such as molecular chaperones, gene therapy, and the treatment with stem cells.[35,37,52,59]

4.1 Enzyme Replacement Therapy

The ERT was introduced in 1991. At that time, it was a very expensive approach based on the administration of a recombinant active protein, which, after internalization into the cells, replaces the natural non-functional GCase. This approach was first suggested by Brady,[60] who observed and quantified the level of GCase in GD patients and normal individuals, recognizing the need for implementing the enzyme from an external source in the case of the disease. The first adopted enzyme was Aglucerase (Ceredase, Genzyme Corp.) deriving from placental cells. Thereafter, Imiglucerase (Cerezyme, Genzyme Corp.) was approved; it was produced by ovary cells from guinea pig through recombinant techniques. Later on, Velaglucerase alfa (VPRIV, Shire Human Genetics Therapie Inc.), produced from fibroblast cell lines, was approved by FDA. Finally, in 2012 Taliglucerase alfa (Elelyso, Pfizer Inc. in the USA, Protalix, BioTherapeutics in other countries), from carrot cells, was approved. The latter differs from all the previous enzymes for the presence at the N-terminus of two amino acids deriving from the linker used to insert the signal peptide, for the presence at the C-terminus of seven aminoacids deriving from the vacuolar targeting signal, and for the presence of sugars typical of vegetal cells such as fructose and xylose. Imiglucerase and Taliglucerase have quite different amino acid sequences compared to the human protein and Velaglucerase, which share the core portion, however their crystal structures are very similar.[36,58] For the administration, the enzymes are glycosylated in order to expose mannose residues, which are recognized by macrophages, promoting the internalization of the protein and then the translocation to the lysosomes.

The intravenous administration requires variable frequencies and dosages, depending on each individual, and the response is evaluated by measuring improvements of multiple parameters such as blood count, volume of organs, skeletal mineral density, medullary fat, bone crisis, quality of life, pain, chronic fatigue, and growth process (for children). In general, the quality of life greatly improves allowing patients to live almost a normal life. The safety of these biological drugs is good, there are no particular side effects and both the development of specific antibodies and the allergic reactions related to the prolonged and continuous intravenous administration are rare.[37,58,61,62] This therapeutic approach is particularly employed in patients with GD1 and in children. For GD2 patients the ERT is not effective and in GD3 is

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controversial since there is no evidence of its efficacy on the neurologic manifestations, although it can be useful to alleviate the peripheral symptoms.[35]

A recent study suggests a novel approach for the transport across the BBB of the GCase,[63] which is normally not able to reach the CNS due to its protein nature. This method relies on the use of nanovesicles of SapC and dioleyl-phosphatidylserine (DOPS). SapC, with its protective function of GCase, is conjugated with a phospholipid allowing tissue translocation. The obtained nanovesicles are very stable in serum and are easily internalized in the cells through a mannose-receptor independent mechanism, that allows the internalization even in the BBB composing cells *via* phosphatidylserine receptors expressed on the surface of capillary cells and on cerebral cells. The stability of the so-delivered enzyme, as well as its lysosomal internalization, is higher than the free enzyme. The study highlighted an increased enzymatic activity and a reduction of the accumulation of the lipidic substrate, with a consequent amelioration of the symptoms, even at the neurological level.[63]

A common drawback of ERT is its very high cost,[64] which is in part due to the low stability of the recombinant enzyme in blood, causing routinely intravenous administration to obtain an efficient therapeutic effect. An emerging strategy to increase the enzyme half-life, preventing its degradation upon systemic administration, is the use of nanoparticles as enzyme carriers, which can be properly functionalized to release their bioactive cargo to the specific site of action.[65]

In this perspective, glucocerebrosidase has been successfully encapsulated inside non-infectious virus-like nanoparticles (VLP's) functionalized with mannose groups to be specifically targeted to macrophage cells.[66] The encapsulated GCase was efficiently internalized in the cells, resulting in a significant increase of its activity. Moreover, this enzymatic preparation proved to be more stable compared to the free enzyme, thus resulting in a prolonged half-life in the blood stream which, in turn, can reduce the frequency of the injections and consequently the overall cost of the treatment.

4.2 Gene Therapy

Gene therapy requires the introduction of GBA1 gene encoding for the GCase in the hematopoietic stem cells to be administered to the patients, to replace the cells producing the defective enzyme. There are several studies describing the production of enzymes using transduced cells, but their protein synthesis does not seem to be sufficient to provide a permanent cure,[67-69] since the transduced cells do not possess a real proliferative advantage compared to the non-modified ones. Therefore, the amount of secreted enzyme is too low to produce a metabolic improvement with a real therapeutic effect. Viral, retroviral and lentiviral vectors could be used to deliver the gene of β-Glucocerebrosidase in the hematopoietic cells, but at present promising results have been obtained only in rat models.[67,70]

4.3 Molecular Chaperones

Molecular chaperone therapy relies on the administration of small molecules facilitating the correct folding of proteins by providing them with their proper functionality. Very often the mutated proteins undergo an impaired folding with consequent degradation before its translocation to the site of activity.[71] The chaperon binds the protein and protects it, also preventing improper aggregation. In general, the molecular chaperones are competitive reversible inhibitors of the enzyme, and they serve as the support on which the defective protein can correctly fold; however, also molecular chaperones are known that target allosteric binding sites, thus not competing with their natural substrates. Typical examples of molecular chaperones promoting the correct functional folding of the GCase are iminosugars:[72] it has been noted that their administration to fibroblasts of GD patients results in a 2-fold increased residual activity of the defective enzyme.[62] With this aim, several other iminoglucidic GCase inhibitors have been designed, but to date molecules successfully applied in therapeutic approaches are missing.[73] However, studies performed using **Ambroxol** (a pH-dependent, mixed-type GCase inhibitor) in combination with ERT showed good results in terms of reduction of the levels of cerebrosides, increased enzyme activity, and amelioration of the CNS symptoms, such as myoclonic seizures, both in rat models and in restricted studies with patients.[62,72]

4.4 Histone Deacetylase Inhibitors

Some mutations can produce catalytically active but structurally unstable proteins leading to their interaction with chaperone proteins, such as Heat shock protein 90β (Hsp90β), which in turn induce the premature degradation of the protein mediated by the ubiquitin-proteasome system. Diminishing such interaction between GCase and the chaperone could result in an ameliorated symptomatology. The inhibitors of histone deacetylase (HDAC) induce a hyperacetylation of the median domain of Hsp90β, altering the recognition of the mutated GCase whose level within the lysosome (partially) increases along with its functional activity.[74]

An example of HDAC inhibitor is the **Vorinostat** (Fig. 4), which is also active against some tumors, such as multiple myeloma and cutaneous lymphoma of T cells. Since this molecule is able to cross the BBB, its use has been proposed not only for the treatment of GD1 but also to treat GD2 and GD3 forms.[62,74]

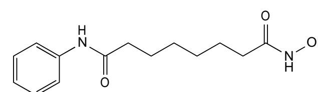

Figure 4. Chemical structure of **Vorinostat**.

5. Substrate Reduction Therapy

SRT was first postulated by Radin[75] in 1984 and then demonstrated in 1994 with the first active molecule against GCS, **Miglustat**. It is based on the inhibition/reduction of the production of glucosylceramides, preventing their accumulation and avoiding the insurgence of GD symptoms.

While the diminished production of sphingolipids results in an amelioration of the pathological outcome of the disease, on the other hand, given the complex role these lipids play within the cells, the physiological impact of their synthesis' inhibition should be taken into account. However, there are no strong evidence of physiological disfunctions or adverse events that can be related to this therapeutic approach. The clinical efficacy of SRT compared to ERT has been reviewed, indicating a similar safety

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profile for both therapeutic treatments and a comparable amelioration of some parameters, such as hemoglobin concentration and platelet count, liver and spleen volume and biomarkers levels (*e.g.* CT, CCL18, Lyso-Gb1).[57,76]

The typical SRT side effects reported during different clinical studies consist of gastrointestinal events such as diarrhea, nausea, vomiting, abdominal pain, attributable to off-target inhibition of intestinal disaccharidases,[77,78] and some neurological events including tremors, convulsion and headache. However no other relevant secondary responses related to the impaired equilibrium of glycosphingolipids level result from the GCase inhibition (when selective), or at least not more serious than disease the SRT is used for.

5.1. Glucosylceramide Synthase

The GCS, also named ceramide glucosyltransferase andavailable in the Uniprot database with the code Q16739, catalyzes the synthesis of glucosylceramides, being very selective for the D,L-*erythro*-ceramide compared to the *threo*-ceramide; the UDP-glucose has been identified as the main hexose donor in this specific reaction,[79,80] with the transfer of glucose to the hydroxylic group on the C1 of the ceramide itself (Fig. 5).

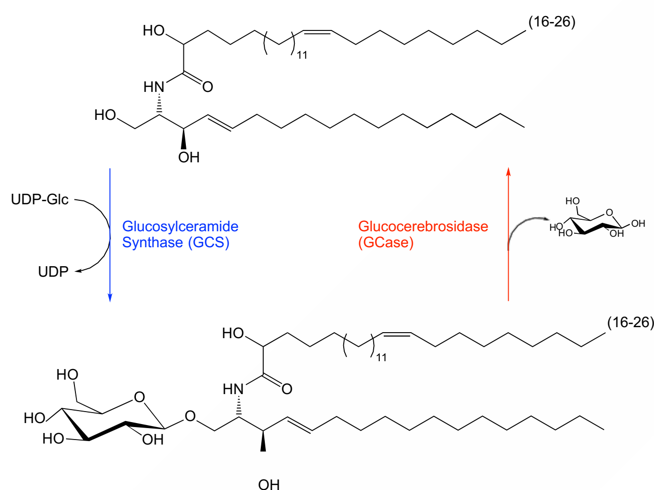

Figure 5. Reactions catalyzed by GCS and GCase.

The GCS usually works at a basal level within the whole organism, but its activity is very low in kidneys, due to the presence of nucleotide-pyrophosphatase enzymes which interfere with its activity. The presence of cations in the reaction system induces a variation of the enzymatic activity: in basic pH condition, magnesium, manganese, and calcium stimulate the synthase activity whereas iron, zinc and copper produce an inhibitory effect, and the optimal pH is 7.4.[81]

The location of the GCS, which is anchored within the membrane of the Golgi apparatus (Fig. 6), differs from that of the Galactosyltransferase, excluding structural analogy with this enzyme.[79] However, several glucosyltransferases within the Golgi apparatus or the endoplasmic reticulum are topologically similar to the GCS, with a conserved cytosolic active site and a transmembrane domain at the N-terminus. It has been shown that the C-terminus sequence and a hydrophilic loop vicinal to the transmembrane domain are accessible from the cytosol. Thus, the orientation of the enzyme toward the cytoplasm allows the synthesis of the glucocerebrosides directly in the cytoplasm.

The GCS, as well as other transferase enzymes, has at least two hydrophobic regions organized within the phospholipidic membrane, both within the N-terminus and after the common hydrophilic domain. It has been suggested the presence of hydrophobic zones beside the C-terminus because both the hypothesized active site, which comprises the characteristic domain of the transferases, and the C-terminus point toward the cytosol. The C-terminus lipophilic transmembrane domains could have a specific function in translocating the products into the lumen of the Golgi apparatus. The results obtained upon treatment of the Golgi membrane with cross-linking agents has highlighted the possibility that the GCS can form dimers or oligomers with different proteins within the membrane itself.[82]

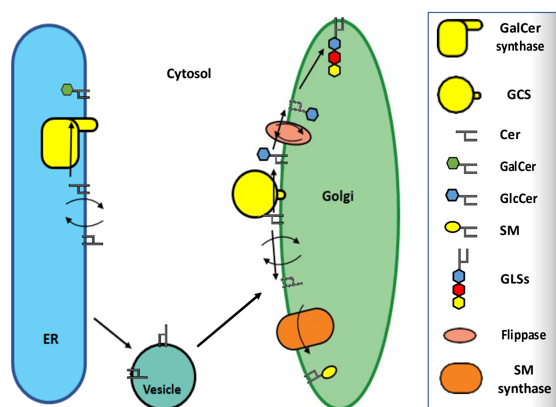

Figure 6. Localization of GCS at the level of Golgi membrane.[79]

The primary structure of the enzyme, comprising 394 amino acids, has been identified, and the sequence of the mouse protein showed a 98% similarity with the human GCS; studies performed on GCS of different species showed a partial similarity with other three homologous protein sequences in the *Caenorhabditis elegans*.[83] By aligning the GCS sequence with sequences of several enzymes which transfer sugars, specific non-conserved residues (His-193 and Cys-207) were identified which determine the binding affinity for the specific sugar.[81] The GCS has been purified with great difficulty because it is strongly tied to the Golgi membrane. Its functional domains remain unknown and the structural diversity, as compared to proteins whose crystallographic structure is known, does not allow a structure homology approach to determine the tertiary structure and its active site.

However, the structure of the enzyme is available in the EBI-AlphaFold repository[84,85] and, at the time of writing, the whole structure is reported with high confidence. This permits to describe quite properly the conformation of some parts of the structure, while for others it not yet perfectly predictable (Fig. 7).

Several amino acid residues, required for the synthase functionality, have been identified such as histidine 193 (His-193), found in the rat GCS as pivotal residue for the binding of the UDP-glucose. Its binding with diethylpyrocarbonate (DEPC), a specific target reagent for amino acids (especially histidines), inactivates the enzyme. Similarly, it has been showed that preincubation of the enzyme with UDP-glucose inhibits the interaction with DEPC, thus highlighting that the interaction between His and DEPC takes

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place in the same site or near to the binding site of the UDP-glucose.[81] To confirm this hypothesis, several recombinant proteins mutated at the His-193 have been produced, which were inactive, showing in these cases the low influence of the DEPC on the enzyme activity, and thus confirming the importance of this amino acid residue in the binding with potential synthase inhibitors. However, it has been not possible to demonstrate that histidine directly binds the activated glucose and that is part of the active site of the enzyme.[81]

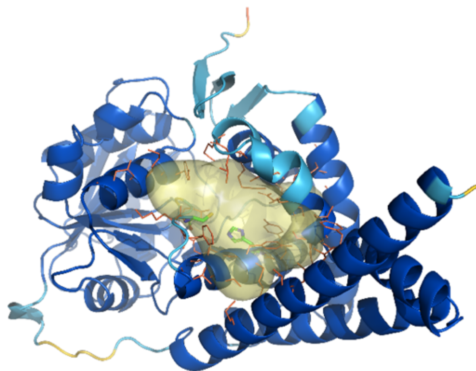

Figure 7. The 3D coordinates of the structure of GCS are the results of a computational prediction (AlphaFold, update Nov 2022).[84,85] The protein is shown with the cartoon style, colored by confidence by means of the "per-residue confidence score" (pLDDT), according to AlphaFold scheme: blue stands for very high confidence, with values larger than 90%, cyan stands for confident (values between 70% and 90%), yellow for low (between 50% and 70%) and salmon very low (below 50%). With the software Flap, with default options,[86,87] cavities were searched, and the first cavity proposed by the software is highlighted within the core of the protein structure. The two residues mentioned through the text, His-193 and Cys-207, are highlighted in green, whereas the other residues that putatively compose the pocket are in orange.

Mutagenesis studies have also identified another important residue, Cys-207, highlighting that the region close to His-193 and Cys-207 includes a characteristic motif (D1, D2, D3, (Q/R)XXRW) which could be part of the active site. Specific mutations within this motif confirmed its pivotal involvement in the enzymatic activity, suggesting that this sequence probably is part of the active site. Since this amino acid sequence is conserved in other glycosyltransferases of the *Glycosyltransferase family 2* (GTF2 family), a plausible hypothesis is that these enzymes share the same catalytic mechanism, while the specificity for the sugar depends on the non-conserved amino acids, as previously mentioned.[81]

The first studies on GCS inhibitors pointed out that there is a general reduction of cellular proliferation as a response to an increased concentration of intracellular ceramides. Studies on Madin-Darby canine kidney (MDCK) cells have evidenced an increased enzyme concentration in the presence of iminosugars or analogues of glucosylceramide such as L-1-phenyl-2-decanoylamino-3-morpholyn-1-propanol (**PDMP**). When performing experiments in the presence of inhibitors of protein synthesis, such as actinomycin, a progressive reduction of the synthase enzyme concentration was noted, due to the physiologic protein turnover, but this effect was slowed down upon using GCS inhibitors.[88] This could be due to an increased stability of the enzyme-inhibitor complex, which renders the protein more resistant to the degradation process. However, the increase of GCS concentration is not caused only by this mechanism since other molecules, like *erythro*-**PDMP** or N-acetylsphingosine, induce the same effect without preventing its catabolism.[89] There are also molecules that increase the concentration of ceramide without altering the concentration of glucosylceramide, others that efficiently inhibit the synthesis of glucosylceramides without varying the concentration of ceramides. The latter, if capable of doing it without inhibiting GCS, display an antiproliferative effect by arresting the cellular proliferation. By using a radiolabeled ceramide, the formation of 1-O-acylceramide was detected,[90] whose synthesis is catalyzed by an enzyme unknown up to the beginning of the last century, the 1-O-acylceramide synthase. This enzyme catalyzes the introduction of an acyl group in position 1 instead of that of a polar group (*i.e.* the sugar) by transferring the acyl group from the phosphatidylethanolamine or from the phosphatidylcholine with a transacylation mechanism.[91] This synthase is located within the lysosomes and belongs to the family of phospholipases A2. Its inhibitors produce an increase of the concentration of ceramides with a consequent antiproliferative activity. Thus, for the treatment of GD with the SRT, it is important to use compounds able to inhibit the GCS without interfering with the activity of 1-O-acylceramide synthase since its inhibition would produce a marked increase in the concentration of ceramide, resulting in cytotoxic effects.

The extensive investigations aimed at designing inhibitors of GCS have led to a hypothetical definition of the active site, which could be formed by three recognition portions: an anionic portion that binds the activated form of glucose, a small region accommodating the linear aliphatic chain of the amide portion and a wider region that interacts with the hydrocarbon chain of the sphingosine. To date, this is the only proposal regarding the topography of the active site of GCS and the lack of more detailed information strongly hampers the design and the development of novel inhibitors of GCS.

5.2 Iminoglucidic Glucosylceramide Synthase Inhibitors

5.2.1 Miglustat

Since GCS possesses a specific binding site which recognizes glucose, one of the first approach to study potential inhibitors of this enzyme has been the use of molecules mimicking the sugar. Iminosugars are known to inhibit the activity of several enzymes as in the case of **Deoxynojirimycin** related compounds (Fig. 8) which inhibits the reaction of N-glycosylation, in particular blocking α-glycosidases. Several studies have demonstrated the ability of these derivatives to inhibit the biosynthetic pathway of glucosylceramides at an early synthetic step, thus affecting the synthesis of a rich series of glycolipids.[92] The N-alkylation of deoxynojirimycin has been extensively investigated confirming that N-butyl-deoxynojirimycin (**Miglustat**, Fig. 8) is a potent inhibitor of GCS.[92,93]

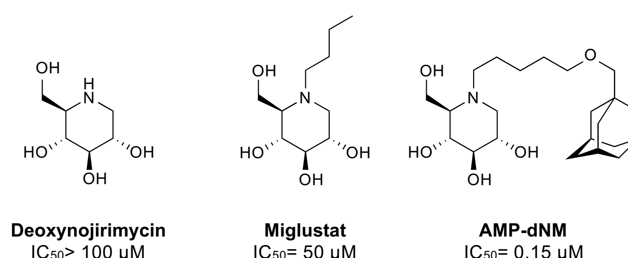

Figure 8. Chemical structures of **Deoxynojirimycin**, **Miglustat** and **AMP-dNM** with their IC_{50} values.[93]

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By increasing the length of the alkyl chain, it has been possible to evaluate the potential involvement of this molecule in the recognition of the enzyme active site, thus acting as a reversible competitive inhibitor toward both the ceramide and the glucose donor, as indicated by the inhibition models derived from the kinetic experiments. Increasing the hydrophobicity of the chain results in an enhanced inhibition potency of the enzyme and in an augmented uptake across membranes and tissues.[94] However, analogues possessing a very long chain display a non-competitive inhibition kinetic and the potential to interfere with the cellular proliferation.[95] Regarding the piperidine ring, present in all **Miglustat** analogues, it requires the configuration of glucose or galactose to inhibit the enzymatic activity.[96] Further optimization studies have indicated that acylation of the nitrogen does not improve the enzyme inhibition activity, whereas the introduction of a sterically hindered alkyl substituent, such as the 2-(hydroxymethyl)-1-[5-(tricyclo[3.3.1.13,7]dec-1-yl-methoxy) pentyl chain produced the active compound **AMP-dNM** (Fig. 8).[97] **Miglustat**, one of the first reported inhibitors of GCS, produces a decrease of the intracellular concentration of glucosylceramide, thus reducing the volume of the spleen and the liver, ameliorates thrombocytopenia, and the quality of life of the patients. It was approved in 2003 for the treatment of GD, and it is highly effective as maintenance therapy post-ERT or in combination with ERT, producing a synergistic therapeutic effect.[98] **Miglustat** is indicated for the treatment of Gaucher patients with a symptomatology from mild to moderate, while is not sufficiently potent for the treatment of more severe conditions. The major side effects include inhibition of the disaccharidase digestion resulting in adverse reactions involving the intestinal system, such as diarrhea (more than 80% of patients) with consequent weight loss, together with tremors and migraine.[99,100] To date, **Miglustat** is considered a second line therapy for GD1, for those patients who cannot be treated with ERT, because of developed resistance to the enzymatic treatment or immune reaction toward the enzyme, and although it can cross the BBB, it does not possess sufficient potency at the CNS level to be used for the treatment of GD3.[101]

5.3 Non Glucidic Glucosylceramide Synthase Inhibitors

5.3.1 Propiophenone Analogues

The 2-amino-3-hydroxypropiophenone scaffold (Fig. 9) was identified by Radin and Vunnam as the core structure of various analogues, structurally similar to glucosylceramide, which acted as inhibitors of GCS in tests performed on rat brains.[102] Several structural modifications have been evaluated to delineate the structure-activity relationship toward the enzyme and to obtain derivatives with improved inhibitory activity. The acylation of the amine by introducing chains of various length was explored, as well as the decoration of the phenyl ring with different substituents, and the variation of the hydroxyl group with new moieties (Fig. 9). Different acyl derivatives of 2-amino-3-hydroxypropiophenone were evaluated, highlighting that the presence of a ten carbon lipophilic chain gives the best results in terms of potency (compound **RV-49**, IC_{50} = 300 μM, 70% inhibition, Fig. 9), measured as percentage of inhibition of the enzyme in comparison with the control, while longer alkyl chains produced adrop of the activity in the *in vitro* enzyme assay.[102]

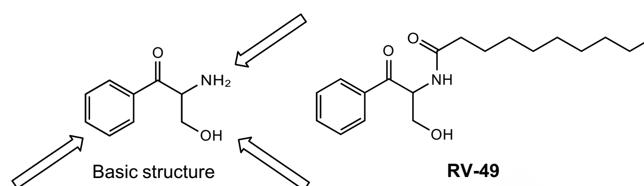

Figure 9. Chemical structure of 2-amino-3-hydroxypropiophenone, and **RV-49**.

The substitution of the terminal carbon of the alkyl chain with a bromine did not affect the activity of the molecule, whereas its introduction in proximity of the amide portion resulted in a detrimental effect. The presence of a double bond on the alkyl chain, as for the (*trans*)-2-decenoyl moiety, further enhanced the activity (79% of inhibition, data not shown). Also, the influence of various substituents at the *para* position of the aromatic ring of the *N*-decanoyl derivative **RV-49** has been evaluated. Small group such as methoxy or methyl did not have great impact on the inhibitory activity, whereas the introduction of more hindered appendages such as chlorine and bromine atoms reduced the efficacy of the molecule (Fig. 10).

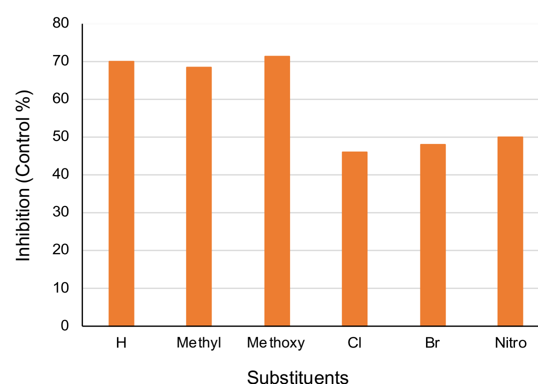

Figure 10. Effect of *para* substituents of the N-decanoyl propiophenone derivatives.[102]

However, propiophenone derivatives bind the enzyme in a covalent manner, forming Schiff bases or adducts with amine groups of lysine residues within the protein sequence, therefore they have not been used as scaffold for the development of novel drugs since they gradually inhibit GCS, inducing its total inactivation one hour after the administration.[102]
This inhibition mechanism has been noted also for those analogues bearing an oxyrane moiety in position 3.[103] The promising activity of the 2-decanoylaminopropiophenone **RV-49** was further explored by preparing several amine analogues among which the D,L-2-decanoylamino-3-morpholino-1-propiophenone (compound **RV-378**, Fig. 11) was effective in reducing the concentration of hepatic GlcCer in mice, whereas it was heavily subjected to metabolism and inhibited Monoamine oxidases (MAO) too.[104]

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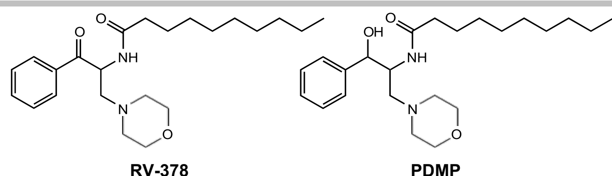

Figure 11. Chemical structure of **RV-378**, and **PDMP**.

5.3.1.1 The First Non-Glucidic Inhibitor: PDMP

Given the interesting but not optimal characteristics of **RV-378**,[104] further optimization approaches were carried out to look for more active inhibitors. The reduction of the carbonyl group led to a diastereomeric mixture with a more active and stable molecule than the propiophenone analogue, the already mentioned **PDMP** (Fig. 11),[105] and later it has been demonstrated that the D-*threo*-(1*R*,2*R*) diastereoisomer is responsible for the inhibitory activity. The kinetic experiments have showed that **PDMP** inhibits the enzyme through a competitive mechanism with respect to the ceramide, thus not forming any covalent bond with the target, and expressing a K_i of 0.7 μM.[105] Although its GCS inhibitory activity is not very strong (IC_{50} = 20 μM), this compound has been very useful for further structure-activity relationship studies, in particular taking into consideration that GD patients often have partially functioning GCase and even a partial inhibition of GCS allows to restore a new equilibrium between synthesis and cellular metabolism of its substrate, avoiding the accumulation of lipid material within the cell.[106] Therefore, this molecular skeleton has been considered a lead structure for the development of more active derivatives.

5.3.1.2 Molecular Core Modifications: PAPD, Phenylalaninol and Norephedrine Analogues

The 1-phenyl-2-amino-1,3-propanediol core **PAPD**, derived from **PDMP**, has been subjected to extensive modifications such as the elimination of the hydroxy group in position 1, obtaining the D-phenyalaninol (Fig. 12) with loss of the chiral center, or the elimination of the hydroxy group in position 3, affording D-norephedrine (Fig. 12). These structures, studied by Warren *et al.*,[103] retain the aromatic ring of the **PDMP**, which mimic the hydrophobic portion of the sphingosine, while several acyl chains of various lengths have been introduced on the nitrogen in position 2, demonstrating that the steric hindrance at this position does not have great effect on the inhibition of GCS, as it does on the inhibition of galactosyltransferase.[103]
Relatively small effects, in terms of inhibition of the enzyme, were observed within the series of amide derivatives of **PAPD**. These compounds bearing a *para*-nitro group on the phenyl ring showed more promising results with the **D-threo-PAPD** decanoyl derivative, displaying a percent of GCS inhibition of 44% with respect to the control. In general, a marked sensitivity to the configuration of the C1 carbon atom was observed within the series with amides of ***threo*-PAPD**, acting as inhibitors, while the amide of ***erythro*-PAPD** stimulated the enzyme.[103]
The modification explored in the case of phenylalaninol scaffold produced some interesting derivatives, indicating that the removal of the C1 hydroxyl group enhances the inhibitory effect. The decanoyl derivative, once more, displayed a good inhibitory activity (39%) and its analogue bearing a methyl group at the C2 carbon atom was found to display an enhanced effect (56% of inhibition).[103]

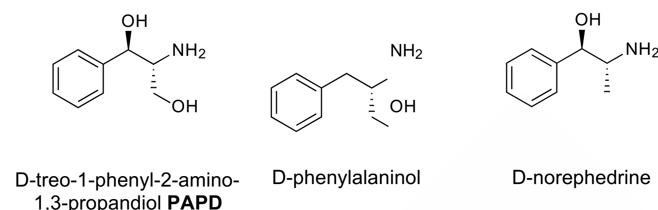

Figure 12. Core structure of **PAPD** and some variations.

Finally, the best inhibitors of GCS were characterized by the absence of the C3 hydroxyl group, as in the case of derivatives of norephedrine. A distinct chain length effect was observed in this series with the *N*-decanoyl DL-norephedrine analogue showing the best inhibitory profile (69%).[103]

5.3.1.3 PDMP Derivatives Chain Length Modifications

The **PDMP** has played a pivotal role in the comprehension of the non-sugar-like competitive GCS inhibitors family. By synthesizing a large number of analogues bearing various functional groups, such as alkyl chains with different length, it has been possible to gather important information regarding the GCS active site. The experiments carried out on entire cells have highlighted that inhibitors bearing longer chains are internalized better than compounds with short chains, thus partially explaining the substantial difference in the inhibitory effect observed intracellularly. For instance, the percentage of inhibition for **PDMP** (10 carbons chain) at a concentration of 5 μM is 16% while in the case of the derivative bearing a 16 carbons chain (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, **PPMP**, Fig. 13) the inhibition is 32% at the same concentration and in the same *in vitro* experiment setup. **PDMP** resulted ten times less active than the analogue with a longer chain, the 1-phenyl-2-myristoylamino-3-morpholino-1-propanol (**PMMP**), which resulted more efficient in slowing cell growth in tumor cells. However, the *in vitro* cellular assay is not as representative as the *in vivo* assay, in which the pharmacokinetic of these derivatives is crucial to understand their specific activity in complex organisms.[107]

5.3.2 Morpholinosphingolipids

Since a possible catalytic active site of GCS has been hypothesized accommodating the ceramide and the UDP-glucose, several compounds have been designed with the ability to mimic both the presence of a sugar analogue and of a ceramide-like moiety. So far, the activities of different 1-morpholino-1-deoxyceramide derivatives have been investigated. The introduction of the morpholine moiety in place of the primary hydroxy group of the sphingosine should mimic the partial positive charge of the transition state formed during the enzymatic reaction. Moreover, since it is known that only one specific configuration of **PDMP** produces a good inhibitory activity, all the four stereoisomers of the 1-morpholino-deoxyceramides have been synthesized and evaluated for their inhibitory activity on GCS, demonstrating that the most active is the 2*R*,3*R*-isomer (Fig. 13), which has the same absolute configuration of **PDMP**, displaying an opposite configuration at one chiral center with

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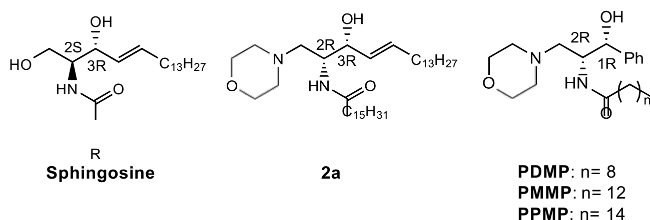
respect to the natural 2*S*,3*R*-substrate (Fig. 13). The most active diastereomer (**2a**, Fig. 13) was the most powerful inhibitor of GCS (73% of inhibition at 5 µM), being more potent than **PDMP** (16-20% of inhibition at 5 µM).[108]



Sphingosine **2a** **PDMP**: n= 8
PMMP: n= 12
PPMP: n= 14

Figure 13. Chemical structure of sphingosine, morpholinosphingolipid **2a**, and other phenyl morpholino derivatives, with chains of different lengths.

5.3.3 Pyrrolidine Analogues of PPMP

Several analogues of **PPMP** have been synthesized introducing different cyclic amines in position 3, replacing the morpholine ring, and retaining the specific stereochemistry of **PDMP** and **PPMP** (Fig. 14).[109] The introduction of a piperidine ring in place of the morpholine of **PPMP** resulted in a better inhibition of the enzyme (12.4% measured as the percentage of GCS inhibition at 5 µM). Superior homologues of piperidine have a detrimental effect on the activity whereas the presence of a pyrrolidine leads to a significant increase of the activity (50% of inhibition) as well as the substitution with an azetidine group, even though in this case with a very limited effect (15% of inhibition). The introduction of a primary amine is not effective, as well as the introduction of an *N*,*N*-diethylamine or of more sterically hindered groups such as *N*-phenylpiperazine.[109]
The functionalization of the aromatic ring has been explored as well, and the introduction in *para* position of a fluorine produces an inhibition decrease; on the contrary, the presence of a methoxy group induces an amelioration of the activity.[109]

R	Activity
	>
	<
	>>
	>>
NH_2	inactive
	<<
	<

Figure 14. Evaluation of the activity of **PPMP** analogues, where the morpholine was substituted with other amines.[109]

The 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol derivative (**P4**, Fig. 15) strongly inhibited GCS with an IC_{50} of 5 µM *in vitro* for the mixture of the four isomers, while the D-*threo* compound showed an IC_{50} of 1.4 µM, as reported by Shayman,[91] while other authors obtained better results (IC_{50} of 0.5 µM).[110]
Another change, as the replacement of the phenyl moiety with the introduction of the sphingosine aliphatic chain, further increased the inhibition at 5 µM up to 87%, as already seen for the pyrrolidinosphingolipids.[105]
Ultimately, a study focused on **P4** evaluated the importance of the hydrophobic alkyl chain in the inhibitory action toward the enzyme.[110] Its complete removal resulted in an inactive compound (**PAPP**), whereas the presence of hexanoyl and decanoyl appendages produced derivatives (**PHPP** and **PDPP**, respectively) with an inhibitory activity 10-fold higher than **PDMP** (IC_{50} = 2.3 µM and 2.4 µM respectively, with respect to IC_{50} = 23 µM for the latter), but still inferior to that of **P4** (IC_{50} = 0.5 µM). Among the tested compounds, the D-*threo*-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol (**PBPP,** Fig. 15) was the most potent GCS inhibitor, with an IC_{50} of 0.3 µM.[110] Moreover, in the case of **PBPP**, no variation of the vitality and cellular proliferation were detected even at high concentration of the compound (160 µM).[110]

Compound	IC_{50} (µM)	Compound	IC_{50} (µM)
P4 ($C_{15}H_{31}$)	0.5	**POPP** (C_7H_{15})	2.2
PAPP (NH_2)	inactive	**PDPP** (C_9H_{19})	2.4
PHPP (C_5H_{11})	2.3	**PBPP**	0.3

Figure 15. Chemical structures of compound **P4** and its analogues substituted in the acyl portion with their activities.[110]

5.3.4 Eliglustat (GENZ-112638)

Following the identification of **P4** and based on the observation that the inhibitory activity further increased in the case of the derivative with a methoxy group in *para* position of the phenyl ring, an additional optimization study was performed. Several substituents differing in electronic (σ) and hydrophobic (π) properties have been introduced on the aromatic ring, and the results indicated an exponential relationship between the IC_{50} of each inhibitor and the sum of the electronic and hydrophobic parameters. In fact, it was found that more negative the values of σ and π, higher the inhibition activity, so aromatic electron-donating and hydrophilic substituents promote the inhibition of GCS. Specifically, the **D-*threo*-4'-hydroxy-P4** derivative (σ = - 0.37, π = - 0.67) displayed an increased activity compared to **P4** (IC_{50} = 90 nM *vs* 500 nM, respectively) and was also more active than the *para*-methoxyphenyl derivative (IC_{50} = 90 nM *vs* 200 nM, respectively).[111]
In addition, several dioxyphenyl derivatives such as the methylene-, ethylene-, and trimethylen-dioxyphenyl homologues, were designed and tested identifying the D-*threo*-1-(3,4-ethylenedioxyphenyl)-2-(palmitoylamino)-3-(1-pyrrolidinyl)

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propanol (**EtDO-P4**, Fig. 16) as the most active inhibitor of this series, displaying an IC_{50} of 11 nM.[111] The active derivatives had little effect on the 1-O-acylceramide synthase activity, whose inhibition leads to apoptosis, thus representing real potential candidates for the GD treatment.[111]

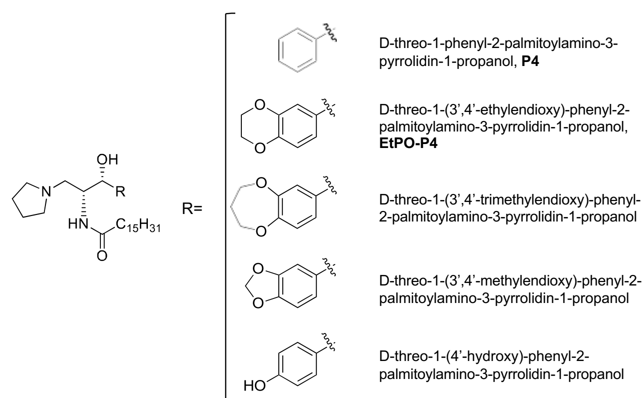

Figure 16. Analogues of compound **P4** and structural modifications of the phenyl ring.[111]

The optimization studies carried out on **P4** permitted to obtain a novel structure presenting the 3,4-ethylendioxyphenyl moiety and the octanoyl chain as substitutes of the phenyl and the palmitic chain, respectively, naming this compound **Eliglustat** (Fig. 17) endowed with an IC_{50} of 20 nM on MDCK cells.[112]

The compound, in its tartrate salt form, has entered clinical trials, demonstrating superior stability and bioavailability compared to the free base.[113] Moreover, it exhibited a pharmacological effect similar to that obtained with **Imiglucerase** ERT. Its potential use as therapeutic treatment has been highly encouraged due to its activity, enzyme selectivity, and the absence of typical side effects observed with **Miglustat**.[114]

The compound was approved by FDA in 2014 for the treatment of GD1, considering that it is not able to efficiently enter the CNS.The superior homologue **Genz-123346** (Fig. 17), having an additional carbon atom on the lipidic chain, is a potent pharmacologic tool designed to reach the CNS, with an even higher selectivity for GCS than **Eliglustat** over α-glucosidases and 1-O-acylceramide synthase.[115]

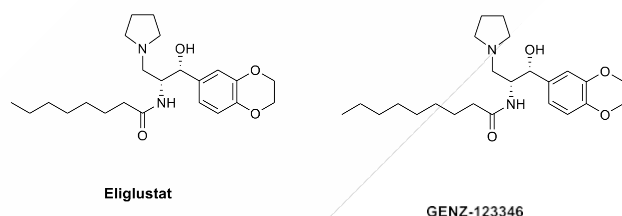

Figure 17. Chemical structures of **Eliglustat** and its superior homolog **GENZ-123346**.

5.3.5 Compounds Active in the CNS

As already mentioned, one of the main problems in the GD therapy, both for ERT and SRT, is the difficulty of an efficient drug delivery into the CNS, precluding the possibility of a therapeutic treatment for patients with cerebral symptoms, a limitation common to **Miglustat** and **Eliglustat**. Moreover, these molecules are subjected to the effect of the glycoprotein P MDR1, a transport protein functioning as an efflux pump removing toxins and xenobiotics from the CNS.

The rational design of novel active GCS inhibitors, able to cross the BBB, has been set up by comparing the differences regarding the main physicochemical properties between the novel inhibitors and drugs known to be active in the CNS. The considered parameters are the topological polar surface area (TPSA), the number of hydrogen bond donors and acceptors (HBD and HBA respectively) and the number of rotatable bonds. Thus, the structure of **Eliglustat** has been tuned according to these parameters, introducing modifications enabling to fulfil the physicochemical requirements necessary to cross the BBB while retaining the pharmacophoric features of the parent molecule. The main modifications were focused on the number of rotatable bonds and on the TPSA, while maintaining the optimal stereochemistry.[116]

Firstly, several modifications of the acyl chain have been evaluated, to reduce the polar surface area and the flexibility of the chain itself. The compounds were evaluated for the enzyme inhibition activity, for their ability to penetrate cells, and for their potential interactions with MDR1 protein.

The first modification has been the decoration of amide side chain with a phenyl ring, obtaining a reduction of the number of rotatable bonds, while the TPSA has been decreased by methylation of the amide function itself. Thereafter, the substitution of the phenyl ring with a more sterically hindered 2-indanylmethyl moiety gave compound **CCG-203586** (Fig. 18), resulting in an ameliorated activity with an IC_{50} of 27 nM on the crude enzyme and of 15 nM on intact cells, respectively, comparable to that of **Eliglustat** (20 nM), even in cells expressing MDR1.[116,117]

CCG-203586 has been screened *in vivo* in mice at different concentrations, showing an inhibitory activity not only atperipheral but also at central level, paving the way to the therapyof GD2 and GD3.[116,118]

The indanyl derivative is subjected to rapid metabolism and clearance, which in turn strongly reduces its presence at the cerebral level. Therefore, the following studies have been focused mainly on modifications of indane and aromatic portions, which are the most subjected to the metabolism reactions, and secondly on the pyrrolidine ring.

The modification of the bicyclic indanyl structure led to a drop of activity without improving the stability against the microsomal metabolism (Fig. 18).

X	R_1	R_2	crude enzyme IC_{50} (nM)	MDCKs cell IC_{50} (nM)	MLM $T_{1/2}$ (min)[a]	
CH_2	H	H	27	15.3	2.5	**CCG-203586**
C=O	H	H	193	>30[b]		
CH_2	H	F	14	1.3	1.1	
CH_2	F	H	36.5	7.2	2.6	

Figure 18. Investigation on the indane structural variations.[117] MDCKs cells were used for these experiments. [a]Half-life when incubated with mouse liver microsome (MLM). [b]28% inhibition at 30 nM.

On the other hand, the substitution of the ethylendioxyphenyl portion with a 3-fluoro-4-methoxyphenyl moiety in the **CCG-203586** did not affect the activity but at the same time did not improve the metabolic stability. Based on these results, the authors confirmed that the presence of electro-donating groups in *para* position of the aromatic ring is crucial for the activity.[117]

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Another interesting derivative is the one bearing a trifluoromethoxy group in position 4 of the aromatic ring, **CCG- 222628** (Fig. 19). Despite its lower activity in the enzymatic assays on the isolated protein (IC_{50} = 110 nM), it showed a marked improvement when tested in the cell assay (IC_{50} = 3.6 nM), displaying a 7-fold higher activity than **CCG-203586**. In addition, the compound was more stable toward the microsomal oxidation

(Human liver microsomes HLM $T_{1/2}$ 84 min for **CCG-222628** vs 28

min for **CCG-203586**). The pharmacokinetic studies have highlighted a good concentration of the compound in the CNS, thanks to a reduced susceptibility to MDR1-mediated efflux, related to the lower TPSA value (62 for **CCG-222628** *vs* 71 for **CCG-203586**). *In vivo* studies performed on GD mouse have demonstrated that the compound was superior to **Venglustat** (see later) in terms of dose required to produce the same reduction of the cerebral lipids' concentration.[117]

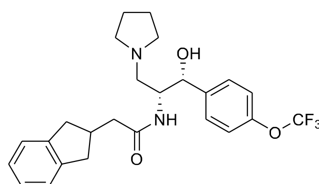

Figure 19. Chemical structure of 4-trifluoromethoxyphenyl derivative **CCG-222628**.

The replacement of pyrrolidine with 3-fluoropyrrolidine or azetidine leads to less active derivatives despite the stability towards metabolism remains almost the same and the compounds are less susceptible to *N*-dealkylation. In this way, it has been possible to demonstrate that the dealkylation is a secondary metabolic mechanism, taking place when the benzodioxane structure is not present.[117]

5.3.6 T-036 and its Optimized Derivatives

Recent studies reported on the discovery of a new class of GCS inhibitors, obtained by means of high throughput screening (HTS) using a cellular assay measuring the decrease of GlcCer in fibroblasts of GD patients.[119,120] The compounds present a novel chemical structure, distinct from the substrate mimetic inhibitors reported till now. The ligand-based SAR study that allowed to obtain an optimization of the structures starting from **T-036** and its derivatives revealed new key pharmacophores for the GCS inhibitory activity. In particular, a compound bearing the bicyclic system 2,3-dihydro-1*H*-pyrrolo[3,4-*c*]pyridine-1-one was identified as hit compound, displaying good properties in terms of inhibitory activity (GCS, IC_{50} = 17 μM), lipophilicity, and solubility. Then a SAR study of the aryl groups (rings A and B, Fig. 20) revealed that an aromatic ring (A) attached at the position 4 of the core, a lipophilic substituent in *ortho* position on ring A, aromaticity of ring B, its coplanarity with the central core, and a *para* substituent on ring B are crucial to obtain potent GCS inhibitors. Furthermore, the trifluoroethoxy group in ring A *ortho* position enhanced the potency, whereas the introduction of a tertiary alcohol moiety in *para* position of ring B improved the pharmacokinetic profile of the molecules. All these findings resulted into preparation of compound **T-036**, as mentioned earlier, a potent GCS inhibitor with an IC_{50} value of 31 nM (Fig. 20).[120]

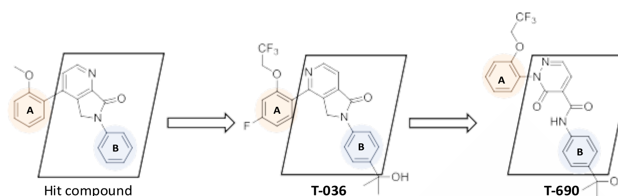

Figure 20. Chemical structures of the hit compound, of **T-036** and **T-690**, with the indication of the major features necessary for the activity.[120]

Interestingly, **T-036** binds the enzyme independently of the concentration of its substrates (*i.e.*, UDP-glucose and ceramide), inhibiting the enzyme in a noncompetitive mode. This behavior contrasts with the binding mode of the other existing GCS inhibitors, like **Eliglustat**, which do not bind to GCS without UDP-glucose, blocking the enzyme activity in an uncompetitive mode against UDP-glucose.

In *in vivo* pharmacological evaluation, **T-036** showed a reduction of GlcCer level in the cerebral cortex and plasma and the pharmacokinetic data clearly showed brain penetration of the compound with a brain:plasma ratio calculated as 0.35. Therefore, the authors have highlighted that the ability of this compound to penetrate the brain could result in a reduction of GlcCer in the CNS. In addition, the effect of **T-036** on Glucosylsphingosine (GlcSph), which plays a role in GD progression and is elevated in neuropathic GD models, have been evaluated. Although the acute administration did not significantly affect the level of GlcSph in either the brain or the plasma, multiple administrations of **T-036** for 2 months significantly reduced GlcSph, with potential therapeutic benefits to neuropathic GD. The authors suggested that, since **T-036** is orally available and brain-permeant, it could be effective in treating the neurological symptoms of GD2 and GD3, therefore representing a promising lead compound.[120]

Further optimization steps were carried out in order to reduce the off-target activity of **T-036** against the serotonin transporter (SERT), that was correlated with the body weight reduction of mice observed during the *in vivo* tests.[119] To this aim, the researchers considered the replacement of the bicyclic core with a single cycle, but since the planarity between the core structure and ring B is crucial for the potency, they introduced an amide linker able to form a hydrogen bond between the core ring, inducing a planarization of the scaffold (Fig. 20). The pyridazine-3-one moiety was evaluated, with the carbonyl, as hydrogen bond acceptor, and the amide moiety, as hydrogen bond donor, so forming a pseudo-six-membered ring. Moreover, the *para* fluorine substitution on ring A was removed, not being essential neither for potency nor for metabolic stability, thus obtaining compound **T-690** (Fig. 20).

Interestingly, **T-690** showed an improved GCS inhibitory activity compared to **T-036** (IC_{50} = 15 *vs* 31 nM, respectively), an improvement of the off-target profile since it had no SERT inhibition activity (IC_{50} > 10 μM *vs* 0.31 μM, respectively), good *in vitro* metabolic stability (clearance in incubations of mouse liver microsomes (μL/(min mg protein): 6 *vs* 1 respectively) and, more importantly, a significantly improved brain penetration (concentration of unbound drug in brain $C_{u,brain}$ 0.24 μM *vs* 0.21 μM, respectively) and a good brain exposure. All these are important parameters when considering the potential use of this

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putative drug candidate for the treatment of neuropathic forms of GD. Moreover, *in vivo* pharmacodynamic assessment showed that **T-690** reduced GlcCer concentration in the plasma and in the brain in a dose-dependent manner.[119]

So, the features of these two novel compounds could represent an interesting therapeutic option for the SRT in neuropathic forms of GD and in an attempt to enhance the biological activity of this new chemical series and to further improve the efficacy of **T-036**, Wang and co-workers conducted an optimization study of the series, investigating the SAR at the 6-position of the pyrrolopyridinone core.[121] A late-stage functionalization strategy employing photocatalyzed iodine-promoted decarboxylation alkylation of heteroaromatic rings allowed the introduction of a variety of substituents at the 6-position of the fused pyridine ring, resulting in a number of **T-036** analogues which enabled the researchers to rapidly expand the SARs of the previously reported series of GCS inhibitors. The introduction of a methyl group and a methyloxetane substituent (compounds **T-036-a** and **T-036-b**, Fig. 21) resulted in two novel derivatives with an improved GCS inhibition potency (IC_{50} = 5.9 nM and 3.6 nM, respectively) compared to the parent compound **T-036** (IC_{50} = 31 nM). These results corroborated the hypothesis that a region with hydrophobic character is present in the ligand binding pocket of the GCS, and it is able to accommodate these types of substituents. Because of their consistent *in vitro* GCS inhibition profile, **T-036-a** and **T-036-b** were evaluated in pharmacokinetic (PK) and pharmacodynamic (PD) studies. Compound **T-036-b** displayed lower CNS exposure compared to **T-036-a** ($C_{u,brain}$, concentration of unbound drug in brain = 0.7 nM and 43 nM at 10 mg/kg dosing, respectively), with **T-036-a** showing an improved GCS inhibitory activity and good brain penetration ($C_{u,brain}/IC_{50}$ (mouse) = 7.8). *In vivo* PD study of **T-036-a** in comparison with **T-036** showed that, after oral administration (10 mg/kg) in C57BL mice, the optimized derivative produced a statistically significant decrease in the concentration of GlcCer in the brain cortex, with an *in vivo* $C_{u,brain}/IC_{50}$ (mouse) of 1.6 and 0.78, respectively. In addition, **T-036-a** showed an evident increase of *in vitro* potency and a superior *in vivo* efficacy than the previously reported compound **T-036.**[121]

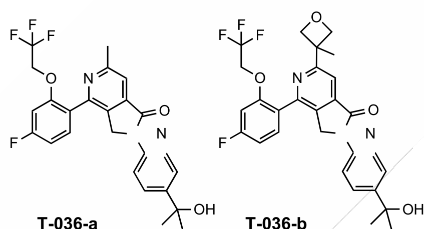

Figure 21. Chemical structures of optimized derivatives **T-036-a** and **T-036-b**.[121]

5.4 GCS Inhibitors with Peptide Structure

5.4.1 Arylethers

The screening of chemical libraries performed by Exelixis company pointed out some interesting peptide-based compounds (Figure 22), in particular a benzylserine derivative bearing in R_5 a chlorine atom, the R enantiomer of which displays a GCS inhibitory activity of 32 nM.[122] Starting from this compound, several analogues with an arylether structure have been synthesized with the aim of studying the importance of substituents of the phenoxypyridine structure, which resulted crucial for the activity. The modifications of the pyridine ring caused a 10-fold drop of the activity. The substitution of the chlorine atom with a methyl group in *para* position of the phenyl ring gave good results (IC_{50} = 53 nM), whereas the decrease of steric hindrance in position R_5 with the introduction of a fluorine reduced the activity (IC_{50} = 300 nM), or dramatically changed the molecular performances as in the presence of hydrogen (IC_{50} = 1150 nM). The *meta* substitution had a detrimental effect on the activity while, on the contrary, an *ortho* substituent ameliorated the activity of one order of magnitude (R_3 = Cl, R_5 = Cl, IC_{50} = 2 nM).[122]

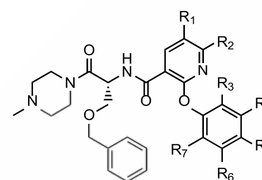

Figure 22. General structure of the arylether derivatives studied in Ref.[122]

The best compound derived from the optimization study of the aryl portion of the scaffold bears the [2-chloro-4-(trifluoromethyl) phenoxy]-2-pyridinyl moiety (GCS IC_{50} = 1 nM); starting from this compound several modifications of the benzylserine portion have been explored. The cytochrome CYP3A4 inhibition and the microsomal oxidation stability studies, carried out on the newly synthesized compounds, showed that the best derivative among those screened is the [2-chloro-4-(trifluoromethyl) phenoxy]-2-pyridinyl one (CYP3A4 IC_{50} (nM) = 13500, mouse liver microsome stability (% conversion): 50%). For this reason, it has been chosen for further optimization studies of the *N*-methylpiperazine fragment, replaced with different amines. Some modifications, such as the introduction of a quinuclidine nucleus, produced interesting results, but the best substituent in this position was the *N*-methyl-aminopyrrolidine moiety. The so-obtained compound showed good activity (GCS, IC_{50} = 16 nM), little inhibition of CYP3A4 (IC_{50} = 12000 nM) and good stability against the microsomal metabolism (mouse liver microsome stability (%conversion): 20%), with a pharmacokinetic and pharmacodynamic profile similar to that of **Eliglustat**.[122]

5.4.2 Cyclopropane Derivatives

Exelixis also identified another peptide-based hit, in this case with the tryptophan residue replacing serine and a cyclopropane substituting the pyridine ring. Further optimization studies have been carried out on this novel structure finding out that, in this particular case, the active stereoisomer is the (*S*)-enantiomer (structure **A**, GCS IC_{50} = 10 nM, Fig. 23).[123] However, the molecule, even if it inhibits very little CYP3A4, does not have an optimal ADME profile, being metabolically unstable (Mouse liver microsomal oxidation, MLMO (% conversion) = 90%, CYP3A4 IC_{50} (nM) = 10000). Thus, several structural modifications have been performed on the tryptophan core, by replacing it with diverse amino acid residues without any real improvement in the activity. Nevertheless, the benzylserine derivative displayed an increased stability toward the metabolic reactions (GCS IC_{50} = 90 nM, MLMO (% conversion)= 50%). The phenylalanine analogue,

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although not very active and with an analogous ADME profile, has been subjected to further modifications with the introduction of substituents on the phenyl ring. These modifications resulted in an increased activity and a higher plasma concentration of the synthesized compounds but, on the other hand, in a higher inhibitory activity of cytochrome and a diminished stability toward microsomal metabolism.

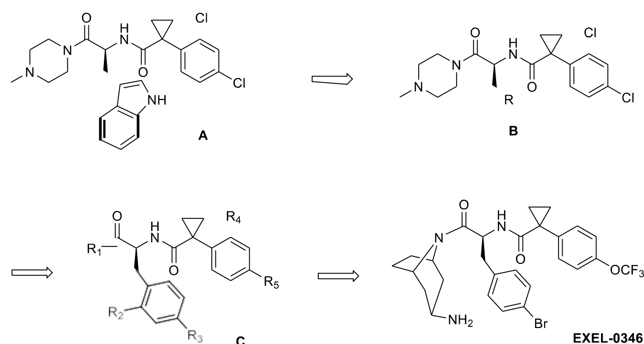

Figure 23. Chemical structures of the hit compound (**A**), derivatives with different structural modifications (**B**, **C**) and their optimized congener **EXEL-0346**.

Only the introduction of a bromine in position 4 of the phenyl ring of phenylalanine resulted in a compound with good potency and with low inhibition of CYPs, but still unstable to microsomal metabolism (GCS IC_{50} = 7 nM, CYP3A4 IC_{50} (nM) = 480, MLMO (% conversion) = 90%). Evaluation of the metabolites has highlighted that the metabolically weak portion of the molecule was the *N*-methylpiperazine. The shift of the methyl with its introduction in position 3 of the piperazine ring resulted in less active compounds. The ring opening as well as the introduction of a 4-aminopiperidine group confirmed the need for a basic amine to retain a good inhibition potency of GCS. The 3-amino-8-azabicyclo[3.2.1]octanyl core has been identified as the best structure in terms of potency for GCS inhibition. The so-obtained compound **EXEL-0346** (Fig. 23) presents good activity toward GCS (GCS IC_{50} 2 nM), and a good pharmacokinetic profile (CYP3A4 IC_{50} (nM) = 2900, MLMO (% conversion) = 7%).[123] Interestingly, **EXEL-0346** has been evaluated in two different diabetes 2 models, with the aim of improving glucose tolerance upon modulation of glycolipids synthesis. Its ability to inhibit GCS in the *in vivo* model caused the reduction of both the levels of GM3, a glycosphingolipid conjugate which interferes with the correct functionality of the insulin receptor, and of glucosylceramides. The latter effect is more important in the case of GD but, to date, the compound has not been yet evaluated in *in vivo* models of the pathology.[123]

5.5 Venglustat (Ibiglustat or ENZ-682452 or GZ-161)

Another molecule deriving from the libraries' screening and optimization processes of carbamic-based structures is the (*S*)-quinuclidin-3-yl-(2-(2-(4-fluorophenyl)-tiazol-4-yl)-propan-2-yl) carbamate **GZ-161** (Fig. 24), which was then named **Venglustat**. *In vivo* studies carried out employing a mouse model of neuropathic GD highlighted the ability of **GZ-161** to reduce the accumulation of glycolipids both at a peripheral and at a central level. In the case of GD2 mouse, the activity of the molecule, although its ability to cross the BBB, produced solely a reduction of the lipids' deposits within the brain, and was not sufficient to normalize the brain levels of glucosylceramide and glucosylsphingosine, which are very high in symptomatic GD2 patients, thus suggesting the role of glucosylsphingosine as a potential contributor to the early death seen in this model. On the other hand, the molecule was useful for the treatment of GD3, in which the lipids accumulation process is slower and the brain levels of glucosylsphingosine are lower in comparison to GD2, inducing a progressive reduction of the amounts of substrate, an amelioration of both the symptomatology and the life expectancy of the patients.[124]

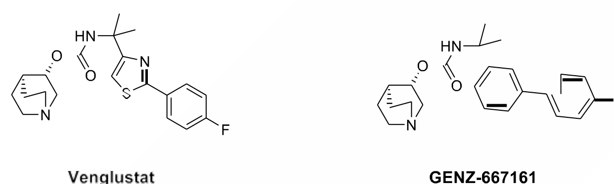

Figure 24. Chemical structures of **Venglustat** and **GENZ-667161**.

The therapeutic potential of **Venglustat** was further evaluated in two different murine models of neurophatic GD, a surrogate model generated by treating mice with coundritol β epoxide (CBE) and the 4L;C* genetic murine model of type 2/3 GD characterized by a significant accumulation of glucosylceramide and GlcSph in the brain and a shortened lifespan. Specifically, in the 4L;C* model **Venglustat** was able to lower the levels of glucosylceramide by 50% and 70% in the brain and in the liver, respectively, compared to untreated mice. Moreover, the brain levels of GlcSph were reduced by approximately 50% upon treatment with **Venglustat**, but in this case the compound was only able to delay the accumulation of GlcSph in the brain, compared to the vehicle treated animals, which despite the treatment continued to increase with age.[125] **Venglustat** (known not only as **GZ-161** but also as **ibiglustat**, **GZ-SAR402671** or **Genz-682452)** received the FDA approval for the treatment of Gaucher disease in 2014. **Venglustat**, together with derivative **GENZ-667161** (Fig. 24),[126] in which a phenyl ring replaces the thiazole ring of **Venglustat**, represents the second generation of GCS inhibitors based on a novel heterocyclic pharmacophore. In fact, also **GENZ-667161** is able to cross the BBB and was shown to reduce the brain levels of GlcCer and GlcSph in a mouse model of neuropathic GD.[127] The administration of these compounds in mice with key deficiencies of GCase, such as the CBE-induced and the 4L;C* models, produced an efficient inhibition of the glycolipids synthesis thus lowering the accumulation of substrates even at a central level, along with visible improvements in histopathological analysis and a 40% increase of life expectancy. Moreover, improvements of cerebral symptomatology such as astrogliosis, microgliosis and ataxia have been observed.[128]

Furthermore, in a phase 2 open-label clinical trial, **Venglustat** has been evaluated in combination with a maintenance dose of the Imiglucerase enzyme replacement therapy during 1 year, in patients with GD3. The clinical study highlighted that during the treatment, the brain concentration of GlcCer decreased below normal range while the GlcSph concentration decreased but did not normalize. In addition, amelioration of ataxia and neurocognitive deficits in GD3 patients have been observed.

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However, the authors of the study do not recommend an off-label use of the compound outside clinical studies.[129]

Finally, in a recent study **Venglustat**, beside its ability to inhibit the activity of GCS, has been shown to competitively bind at the *N*-terminal methyltransferase 1 (NTMT1) substrate binding site, displaying an IC_{50} value of 0.5 μM in both biochemical and cellular inhibition assays. Therefore, it has been selected as lead compound for the development of novel inhibitors of NTMT1 which has physiopathological implications in neurogenesis, retinoblastoma and cervical cancer.[130]

5.6 Pyrazole Ureas as GCS Inhibitors

In search of novel potent GCS inhibitors as therapeutic strategy to reduce glycosphingolipids accumulation in both the periphery, resulting in disorders like Gaucher's disease, and in the CNS, contributing to Parkinson's disease,[131] Roecker and co-workers developed an HTS campaign, which led to the identification of several pyrazole as GCS inhibitors candidates, representing a novel chemotype able to cross the BBB.[132]

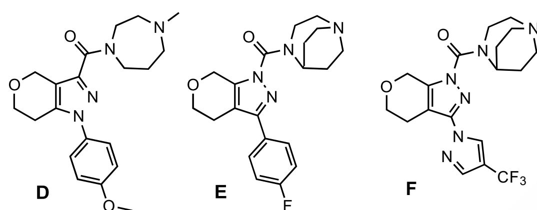

Figure 25. Chemical structures of pyrazole urea derivatives as GCS inhibitors.

The lead compound derived from this screening (compound **D** Fig. 25, GCS inhibition in cells IC_{50} = 180 nM), was subjected to an optimization process based on several design concepts which resulted in the discovery of two novel potent GCS inhibitors, compound **E** and **F** (Fig. 25) displaying higher potency on GCS in cells (GCSi IC_{50} = 3.5 nM and 1.8 nM, respectively) as long as excellent physicochemical properties, including low molecular weight, lipophilicity, and PSA resulting in high central nervous system multiparameter optimization (CNS MPO) and high kinetic solubility across pH ranges. Moreover, both compounds had acceptable CNS exposure to be further employed for *in vivo* studies. Evaluation of *in vivo* pharmacodynamic effects of compound **E** was carried out in a wild type C57 black mice model by measuring brain concentration of GlcCer d18:1/18:0, one of the most abundant GlcCer species in the brain, by LC-MS/MS-based mass spectrometry. Oral treatment with this compound resulted in a significant reduction of GlcCer levels from baseline in a dose-dependent manner both at 30 and 100 mg/kg.[132]

On the other hand, the effects of GCS inhibition on lysosomal hydrolysis and α-syn aggregation were evaluated by studying compound **F** in human induced pluripotent stem cells (iPSC) neurons, which produced a dose-dependent reduction of endogenous GlcCer species, with an IC_{50} value in these neurons of 1.7 nM. Furthermore, to determine whether GCS inhibition correlates with modulation of α-syn pathology, human iPSC neurons were pretreated with different concentrations of **F** a day before treatment with α-syn preformed fibrils (PFFs). The derivative was able to rescue the PFF-induced lysosomal activity deficit and increased lysosomal hydrolysis activity in a dose-dependent manner when compared to a vehicle treated group. In conclusion, compounds **E** and **F** demonstrated *in vivo* pharmacodynamic effects and *ex vivo* improvements in lysosomal function and reductions in α-syn pathology, serving as excellent tools to study GCS biology and the effects produced by its inhibition.[132]

5.7 Other Inhibitors

5.7.1 HA 14-1

HA 14-1 (Fig. 26) is an inhibitor of BCL-2, involved in the apoptosis process of tumoral cells. It mimics the BH3 domain, crucial for the formation of dimers, of this protein interfering with the dimerization of BCL-2 and with its interaction with pro-apoptotic proteins. Several studies have highlighted the ability of **HA 14-1** to inhibit Glucosylceramide synthase displaying an IC_{50} of 4.5 μM without interfering with off-target enzymes.[133] There is no structural homology between GCS and BCL-2 but some degree of homology in the tertiary structure has been hypothesized, in particular between the GCS active site and the BH3 portion of BCL-2.[133]

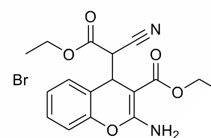

Figure 26. Chemical structure of compound **HA 14-1**.

Thus, several enzyme kinetics experiments have been performed in order to understand the mechanism of inhibition of GCS induced by this molecule, revealing that **HA 14-1** acted as a competitive and mixed type inhibitor with respect to C_6-NBD-Cer and UDP-glucose, respectively, even if it does not share any structural homology with the two substrates.[133] The experiments carried out on cells showed a decreased accumulation of glucosylceramides but also an accumulation of ceramide and cellular death, elements that preclude the use of **HA 14-1** for the treatment of GD.[133]

5.7.2 Hybrid Structures

Several novel compounds have been designed by combining the structural features of iminoglucidic and **PDMP**-related GCS inhibitors. The proposed hybrid derivatives (Fig. 27) present the amino alcohol core of **PDMP** (in black), the iminosugar of **Miglustat** (in blue) or other secondary cyclic amines, and several acyl chains included the adamantane moiety of **AMP-dNM** (in red).[134] Unfortunately, this approach did not produce any active inhibitor of GCS but allowed the preparation of a potent Glucocerebrosidase inhibitor (derivative **G**, Fig. 27, GCS IC_{50} > 10 μM, GBA1 IC_{50} = 2 μM, GBA2 IC_{50} = 90 μM) which could be considered a lead compound for the development of novel active molecules for the treatment of other disorders involving this specific enzyme.

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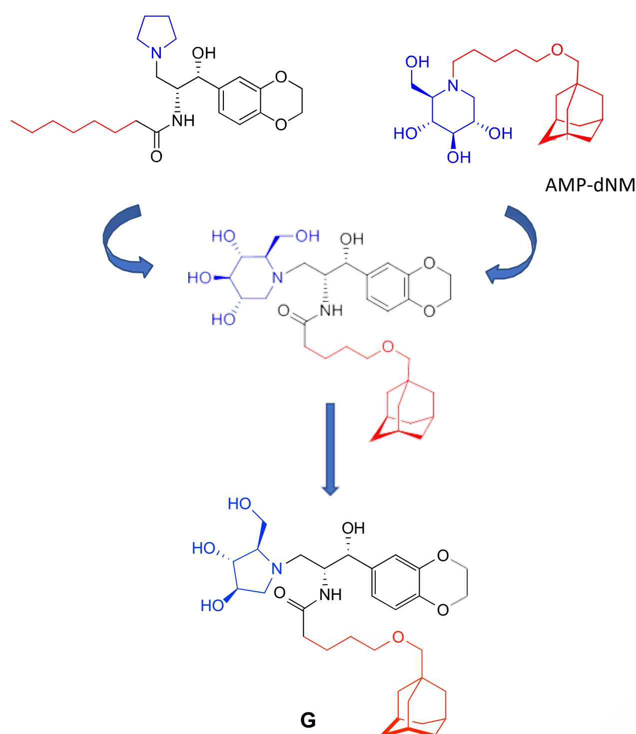

Figure 27. Hybridization approach of **Eliglustat** and **AMP-dNM**, leading to compound **G**.

5.7.3 WP1066

WP1066 (Fig. 28), a compound that inhibits the JAK/STAT3 metabolic pathway, was found to inhibit also the Ceramide glucosyltransferase (IC_{50} = 7.2 μM) with an uncompetitive mechanism,[135] without interfering with the sphingomyelin synthase nor the lactosylceramide synthase. Despite the selectivity displayed against GCS over other synthases, it has not drawn so much interest because of its pro-apoptotic effect and, being an uncompetitive GCS inhibitor, it is active only in the presence of the enzyme-substrate complex. This is an important limitation but, however, its ability to cross the BBB could be a solid starting point for the development of novel GCS inhibitors for the treatment of LSD with neurological symptoms.[126]

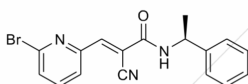

Figure 28. Chemical structure of compound **WP1066**.

6. Alternative Approaches

6.1 Tamoxifen Analogues

The constant research of molecules effective in treating the central symptoms of GD in the neurological forms of the disease pushed studies on fibroblasts from GD2 patients and allowed to identify **Tamoxifen** and **Idoxifene** (Fig. 29) as compounds able to reverse the lysosomal deficit of calcium in these cells.
GD2 fibroblasts are characterized by a reduced release of calcium from lysosomal deposits in response to Gly-Phe-β-naphtylamide (GNP).[127] These compounds increase the concentration of calcium released in response to GNP, with a consequent amelioration of the symptomatology. Since they are very active as antiestrogens, an extensive SAR investigation has been performed, aimed at optimizing their structure to produce molecules effective for the treatment of GD but without off-target side effect on hormone receptors.

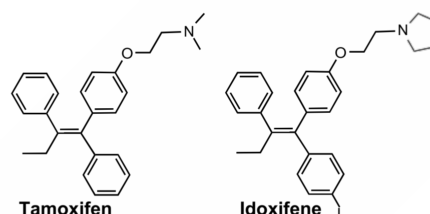

Figure 29. Chemical structure of **Tamoxifen** and **Idoxifene**.

Since it is well known that the hormonal activity of these compounds can be modulated through the introduction of substituents on the aromatic rings not bearing the ethoxydimethylamine function, the optimization process has been primarily focused on structural modifications aimed at minimizing the hormonal effects of these molecules. Thus, substitutions of the aromatic rings, rings simplification, saturation of double bonds and variation of the position of the alkyl group have been investigated (Fig. 30). The elimination of one of the phenyl rings reduced estrogenic activity with a switch in functionality[136] with a reduced release of lysosomal calcium. In general, the modifications evaluated, such as the replacement of a phenyl ring with a benzyl, did not produce an increase of efficacy of this set of compounds.

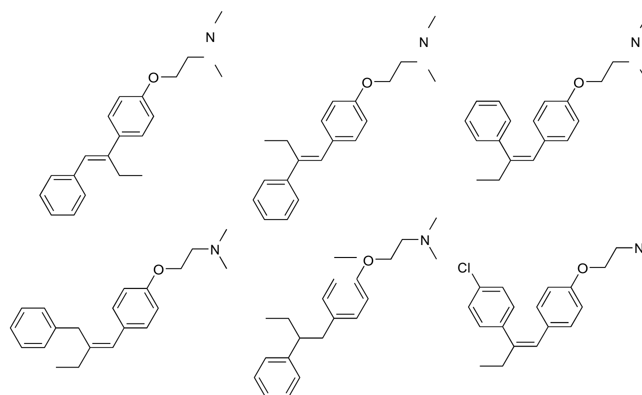

Figure 30. Structural modifications of **Tamoxifen** studied by Childers *et al.*[136]

Based on the hypothesis that less rigid and non-planar structures could result in more active compounds, the optimization process was directed toward diphenylethanoic-like derivatives, retaining

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the dimethylaminoethoxyphenyl portion of the original scaffold (Fig. 31).[136]

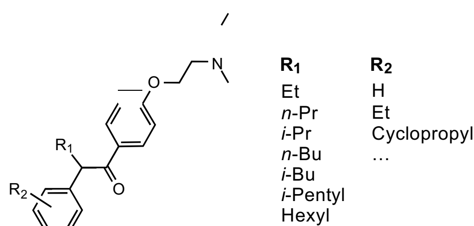

Figure 31. Examples of various appendages introduced on diphenylethanone derivatives.[136]

The modifications of this structure, in particular the substitution of the phenyl ring and the introduction of a substituent in position α to the carbonyl group (Fig. 31), allowed the preparation of several derivatives able to induce the release of lysosomal calcium, E_{max} (percent activation of basal GNP-induced calcium response measured in the absence of compound) = 227-240% of the basal response to GPN in Gaucher cells compared to a value of 50-56% in wild type cells. In particular, the introduction of an isobutyl group in position α to the carbonyl led to a very potent derivative (EC_{50} = 0.9 μM, E_{max} = 108%) while the introduction of an isopentyl substituents produced an increase in potency and efficacy compared to **Tamoxifen** (EC_{50} = 0.9 μM, E_{max} = 191% vs **Tamoxifen**, EC_{50} = 10 μM, E_{max} = 148%). Compounds with a linear alkyl chain such as ethyl, *n*-propyl, or *n*-butyl displayed little effect on lysosomal calcium release from GD patients-derived cells. Thereafter, the introduction of different substituents on the phenyl ring has been evaluated, while retaining the butyl moiety in position α to the carbonyl group. The introduction of substituents like ethyl (EC_{50} = 0.65 μM, E_{max} = 138%), or isopropyl (EC_{50} = 1.4 μM, E_{max} = 401%) moieties in *ortho* of the phenyl ring enhanced the effect by blocking the structure in a specific conformation which reduces the free rotation of the phenyl ring itself. In particular, the introduction of a cyclopropyl in *ortho* position produced a very active molecule (Fig. 32) devoid of estrogen activity (EC_{50} = 3.1 μM, E_{max} = 240%, Estrogen Receptor % Activity @ 10 μM = 0% vs **Tamoxifen** EC_{50} = 10 μM,E_{max} = 148% Estrogen Receptor % Activity @ 10 μM = 100%). Thus, the most active compounds have been selected by evaluating their physiochemical properties and their druggability.The solubility in water and the stability toward the microsomal metabolism have been evaluated and all the selected compoundswere more soluble and more stable than **Tamoxifen**.[136]

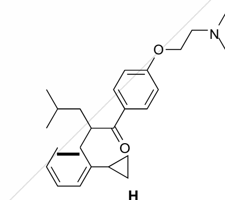

Figure 32. Chemical structure of the optimized derivative obtained upon SAR evaluation.[136]

7. Summary and Outlook

Gaucher Disease is an LSD related with the disruption of glucosylceramide catabolism. It is characterized by a wide clinical spectrum, depending on the affected organs and cell types, and is associated with both peripheral and CNS manifestations.

Considerable efforts have been made over the last 40 years on the development of therapies to treat this glycosphingolipidosis condition. Beside the ERT approach, the development of small molecules able to inhibit the glucosylceramide synthesis mediated by the GCS is one of the most pursued strategies for the GD treatment. Despite this approach has allowed the identification of several active molecules, the GCS localization at the level of the Golgi apparatus has not allowed its adequate isolation and its crystal structure has not been solved yet, thus designing molecules able to interact with its binding site following a rational approach is a challenging task.

The PDMP-related derivatives as well as the iminosugar-like compounds have been designed based on a hypothetical active site. However, this approach does not work for molecules that differ structurally from these two classes of compounds, usually deriving from high throughput screening.

Moreover, none of the mentioned approaches is clearly effective in the CNS manifestation of GD type 2 and 3, whereas ERT is already approved for the treatment of GD type 1. In fact, greater challenges have to be addressed for the CNS-based variants of GD which do not only include the usual difficulties of drug discovery and development but also those associated with rare diseases such as small patient population, prolonged time to achieve clinically meaningful results and limited understanding between genetic abnormality and phenotype.

Therefore, the need of compounds able to treat the symptoms of Gaucher disease, especially at the level of the CNS, has driven the design of novel active compounds as in the case of **PDMP** derivatives which have been modified to produce molecules with the ability to cross the BBB and exert their effects within the brain. This strategy has been applied to the design of **Venglustat**, which has been endowed with the ability to cross the BBB and is one of the most active GCS inhibitors reported to date.

Further potential modifications of this core structure could derive from the combination of different molecular portions identified by HTS, with the ability to cross the BBB, such as the replacement of the phenyl ring with indane-like or arylether-based moieties.

In this way, a hypothetical approach would be to carry out further SAR investigations on the already discovered active molecules to expand the knowledge concerning the composition of the active site of GCS.

In addition, since a specific pharmacophore for the GCS inhibitors has not been yet defined, more information regarding the structure of the active site could be obtained by applying the CoMFA computational approach. In fact, to date important information such as how the already discovered inhibitors interact with the enzyme, which is the role of the specific functionalgroups, which residues of the protein are crucial for the interaction, remain still unknown; the resolution of the enzyme crystal structure will certainly be very helpful for the discovery andthe design of novel active GCS inhibitors for the treatment of Gaucher disease.

In this perspective, Activity-Based Protein Profiling (ABPP) is emerging as a powerful tool for the investigation of structural identity and functions of enzymes within complex biological environment. This approach relies on the use of the so-called Activity-Based Probes (ABP), consisting in a specific motif that

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selectively directs the ABP towards certain enzymes, a chemical moiety that reacts (often irreversibly) with catalytic residues within the active site and a reporter group, enabling detection and/or capture of the ABP after the reaction with the enzyme happened.[137]

ABPs specifically designed toward glycosidases have been developed and largely employed to provide insights into the role these enzymes play in cells.[137,138] Specifically, fluorescent glycosidase ABPs represent an attractive tool to screen libraries of small molecules as potential enzyme inhibitors by using of the high-throughput fluorescence polarization (FluoPol) assay format[137,138] and this approach has been successfully employed to identify selective inhibitors of non-lysosomal glucocerebrosidase GBA2.[139] To date there are no reports on the application of this methodology for the study of GCS principle, but the same FluoPol-ABPP strategy may be applied to study and profile Glucosylceramide synthase, providing important information on the structural composition of its active site and the specific mechanism of action, and this could be really helpful in the discovery process of new GCS inhibitors.

Moreover, for the neuropathic forms of GD, namely GD2 and GD3, the relationship between the accumulating substrate and the clinical manifestation of the disease in not yet well understood and this can hamper the evaluation of agents that could be therapeutically effective. For this reason, the identification of novel and robust biomarkers and their utility in predicting the clinical phenotype and the therapeutic effectiveness of novel agents will be pivotal in the development of new drugs, effective for the neuropathic GD forms, but also will be critical in the design of clinical trials with small number of patients and establishing proof of principle for clinical utility. In this light, mouse models of GD type 2 and 3 are extremely useful in proof of principle studieswhen the therapeutic use of a candidate agent must be assessed.

Abbreviations:

ABP Activity-Based Protein
ABPP Activity-Based Protein Profiling
ACE Angiotensin Converting Enzyme
BBB Blood Brain Barrier
CBE coundritol β epoxide
CCL18 Chemokine(C-C motif) ligand 18
CNS Central Nervous System
DOPS Dioleylphosphatidylserine
ER Endoplasmic Reticulum
ERT Enzyme Replacement Therapy
FluoPol fluorescence polarization
GBA2 Non-lysosomal glucoslylceramidase
GCase Glucocerebrosidase
GCS Glucosylceramide Synthase
GCSi Glucosylceramide Synthase Inhibitor
GD Gaucher disease
GlcCer Glucosylceramides or Glucocerebrosides
GlcSph Glucosylsphingosine
GNP Gly-Phe-β-naphtylamide
GSL Glycosphingolipids
GTF2 Glycosyltransferase 2
HTS High Throughput Screening
IL Interleukin
IRDiRC International Rare Diseases Research Consortium
LSDs Lysosomal Storage Diseases
Lyso-Gb1 Glucosylsphingosine
Lyso-GL1 Glucosylsphingosine
M-CSF Macrophage colony-stimulating factor
MAO Monoamine oxidase
MDCKs Madin-Darby canine kidney
MDR Multidrug resistance
MLM Mouse liver microsomal
MLMO Mouse liver microsomal oxidation
NTMT1 *N*-terminal methyltransferase 1
RD Rare Disease
SapC Saposin C
SERT Serotonin transporter
SRT Substrate Reduction Therapy
TNF Tumor Necrosis Factor
VLP Virus-like nanoparticles

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Keywords: Gaucher Disease • Rare Disease • Glucosylceramide Synthase Inhibitors • Substrate Reduction Therapy

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