MGM_2016_154, revised
Special issue dedicated to the memory of Roscoe O. Brady
Edited by Gregory Grabowski

The metabolism of glucocerebrosides – from 1965 to the present

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Abstract

Gaucher disease is caused by the defective catabolism of the simple glycosphingolipid, glucosylceramide (GlcCer), due to mutations in the *GBA1* gene which encodes for acid β-glucosidase (GCase), the lysosomal enzyme that degrades GlcCer. Today, Gaucher disease patients are routinely treated with recombinant GCase, in a treatment regimen known as enzyme replacement therapy (ERT). We now review the biochemical basis of ERT and discuss how this treatment has advanced since it was first pioneered by Dr. Roscoe Brady in the 1960s. We will place particular emphasis on the three dimensional structure of GCase, and subsequently discuss a relatively new treatment paradigm, substrate reduction therapy (SRT), in which GlcCer synthesis is partially inhibited, thus reducing its accumulation. Both of these approaches are based on studies and concepts developed by Dr. Brady over his remarkable research career spanning six decades.

Key words: Gaucher disease, glucosylceramide, enzyme replacement therapy, substrate reduction therapy

Introduction

The years 1965 and 1966 were seminal for glycosphingolipid (GSL) research as these were the years in which Dr. Roscoe Brady first described the enzymatic cleavage of the simplest GSL, glucosylceramide (GlcCer), and described the biochemical basis of Gaucher disease, which is caused by the deficiency of this enzyme. This critical work was performed in the shadow of the discovery of the function of the lysosome by Dr. Christian de Duve in the 1950s, and the suggestion by de Duve that defects in the activity of the '20 distinct lysosomal enzymes' (the number of lysosomal enzymes that were known in the 1950s) led to a family of diseases known as lysosomal storage diseases (LSDs) [1]. Today, the lysosome is known to contain both soluble hydrolases, along with transporters, activator proteins, structural proteins, ion channels and nutrient sensing machinery. Moreover, the lysosome has become so central to biological and medical research that the Nobel Prize in Medicine for 2016 was awarded to Dr. Yoshinori Ohsumi for his work on autophagy, a process with which the lysosome is intimately connected. Whether a similar accolade should have been giving to Dr. Brady is, sadly, now only a matter of academic debate.

GlcCer degradation by GCase

In 1965 Brady published a series of seminal papers in the Journal of Biological Chemistry, Biochemical and Biophysical Research Communications and in the Journal of Clinical Investigation, in which he, together with Drs. Kanfer and Shapiro, outlined the pathway of glucocerebroside catabolism. Reading these papers over 50 years later is a beguiling exercise. In the first study [2], Brady and colleagues showed that radiolabeled GlcCer was metabolized by an enzyme in human spleen. Although the enzyme was only modestly purified to 82-fold enrichment, this study nevertheless unambiguously determined that the enzyme specifically cleaved GlcCer, rather than galactosylceramide, and demonstrated that the catalytic reaction resulted in the generation of glucose and *N*-

stearoylsphingosine (both of which were radioactively labelled). The second study [3] measured 'the level of the glucocerebroside-cleaving enzyme in human spleen tissue' and showed that there is a 'pronounced diminution of the activity in spleens obtained from patients with Gaucher's disease'. Type 1 Gaucher disease is characterised by hepatosplenomegaly and a variety of haematological defects, and splenectomy used to be a common procedure in these patients. Much of the early biochemical experiments were therefore conducted on spleen tissue. The implication of glucocerebrosidase as the culprit in Gaucher disease was further confirmed [4] when Brady and colleagues also suggested, based on the clinical differences between infantile, adult and juvenile forms of Gaucher disease, that there may be more than a 'single genetic mutation' involved, that is to say, different mutations in the glucocerebrosidase gene might lead to different forms of Gaucher disease. This latter statement is now known to be incorrect and moreover, genotype-phenotype correlations have been notoriously difficult to pin down [5].

As would be expected, huge progress has been made in the study of GSL metabolism and Gaucher disease over the past 50 years (Fig. 1). At present, recombinant forms of the defective enzyme in Gaucher disease (acid β-glucosidase, EC3.2.1.45; GCase), also sometimes known as glucocerebrosidase, glucosylceramidase or GBA1, is routinely generated commercially and administered intravenously to Gaucher disease patients; enzyme replacement therapy (ERT) is hugely successful and more than ~8,000 patients are currently treated worldwide [6]. That is not to say that ERT is without its limitations: indeed, lower efficacy in treating bone and lung disease, along with its inability to cross the blood-brain barrier, render it of limited use in treating CNS manifestations in the forms of Gaucher disease in which the brain is affected, necessitating the development of alternative therapies, such as substrate reduction therapy (SRT), which will be discussed below. In the remaining part of this section, we will discuss the three dimensional structure of GCase and the position

of the mutations in the 3D structure, which has yielded a somewhat disappointing amount of information concerning structure-phenotype correlations.

Remarkably, it was not until 2003 [7] that the first X-ray structure of GCase was reported, that is, ~40 years after the enzyme was first partially purified by Brady and colleagues and over a decade since the enzyme was first used commercially in patients for ERT. Subsequently, a large number of GCase structures were reported, for both the apoenzyme and for the enzyme in complex with various ligands under several experimental conditions (reviewed in [8]). GCase is a member of glycoside hydrolase family 30. The mature protein consists of 497 amino acids and has an M_r of ~62 kDa. The structure comprises 3 non-contiguous domains that could not be predicted from the primary amino acid sequence (Fig. 2). Domain 1 consists of one major 3-stranded anti-parallel β-sheet flanked by a perpendicular N-terminal strand and loop. Domain II consists of two closely associated β-sheets forming an independent domain resembling an immunoglobulin (Ig) fold. Domain III is a $(\beta/\alpha)_8$ (TIM) barrel containing the catalytic site. The function of the two non-catalytic domains is unknown, but the location of mutations throughout all three domains suggests they play important regulatory roles. No clear correlation is apparent between the spatial location of particular mutations and the severity of clinical symptoms. However, some mutations located close to the active site mainly cause severe disease, whereas L444P, which always predisposes to severe disease with a neurological phenotype (i.e. type 2 or 3), is located on domain II distant from the active site (Fig. 2); however, it has proved difficult to determine whether a mutation in one region or domain of the protein will lead to severe or mild disease.

Concerning the catalytic mechanism, Glu235 serves as the acid/base and Glu340 as the nucleophile in the catalytic cycle [7,9]. Two different catalytic mechanisms have been proposed. It was originally suggested that GCase acts like a typical retaining ß-glucosidase for which the catalytic cycle proceeds through a two-step reaction mechanism: a)

glucosylation of the active site by substrate followed by deglucosylation with release of ß-glucose [10]. New findings, however, suggest a different catalytic mechanism due to steric hindrance in the active site. In this mechanism, the anomeric carbon of the glucose cannot be susceptible to nucleophilic attack by Glu340 and, therefore, the glycoside bond must be hydrolyzed via a carbenium ion intermediate [11].

The structure of only one mutation has been verified experimentally, namely the N370S mutation [12]; interestingly, a molecular dynamics simulation [13] came to a similar mechanistic conclusions just a short time before publication of the X-ray structure [14]. Comparison of the experimentally-based structure with the molecular dynamics simulation showed rather small differences between the two approaches, which itself was of interest as it gave some indication of the accuracy of molecular dynamics simulations; both studies reported that the conformation of active site loop 3 changes due to an altered hydrogen bonding network; however, the molecular dynamics study produced additional data concerning the flexibility of loop 1 and the catalytic residues that were not observed in the X-ray study [14].

Finally, the 3D structures of all the three enzymes currently in clinical use have been resolved experimentally (reviewed in [15]) and the structures do not differ in any significant manner. GCase, unlike most lysosomal hydrolases, is not targeted to the lysosome via a mannose 6-phosphate (M6P)-dependent pathway but rather via Limp2 [16]. Uptake of GCase from the circulation into cells is therefore not mediated via M6P receptors but via the macrophage mannose receptor. Various strategies have been developed to maximise mannose exposure of N-glycans on the recombinant enzyme by the various companies who make the ERT products for Gaucher disease. For example, Imiglucerase (Cerezyme®,Genzyme) is produced in CHO cells and undergoes enzymatic glycan remodeling after purification to expose terminal mannose residues [7]; Velaglucerase alfa (VPRIV®, Shire) [17], which is

produced in an engineered human fibrosarcoma cells, is cultured in the presence of kifunensine, a potent inhibitor of the glycoprotein processing mannosidase I, thus maintaining the structure of the newly synthesized N-linked oligosaccharides as immature highly mannosylated structures; the recombinant product is decorated with an array of terminal mannose sugars, and there is no need for treatment with glycosidases; Taliglucerase alfa (Elelyso®, Protalix), which is generated in a high-yield plant (carrot root) cell system [18,19] exploits the fact that plant glycans are dominated by oligomanose structures and therefore does not require inhibitor treatment or glycosidase processing.

In summary, ERT, irrespective of the source of the recombinant enzyme, ameliorates the systemic manifestations of Gaucher disease and is without doubt the most efficacious ERT for any LSD to date. However, while ERT is highly effective in treating type 1 Gaucher disease patients, its inability to cross the blood-brain barrier renders it unsuitable for treating patients with neurological forms of Gaucher disease (i.e. types 2 and 3 disease), although it improves the disabling systemic manifestations in patients with type 3 disease. Thus, additional therapies are needed, such as partial inhibition of GlcCer synthesis.

The biosynthesis of GlcCer

GlcCer is the precursor of almost all GSLs and its biosynthesis represents the first committed step in the GSL biosynthetic pathway [20]. GlcCer is generated by the action of the enzyme UDP-glucosylceramide synthase (GCS, EC 2.4.1.80) which specifically transfers glucose to ceramide [21] on the external leaflet of an early Golgi apparatus compartment [22] (Fig. 3). The suggestion that GCS may represent another therapeutic target for treating Gaucher disease was the vision of Norman Radin [23], the pioneer of what is now termed SRT [20]. The concept is very simple. By partially inhibiting GCS activity, less GlcCer (and therefore also less complex GSLs) would be synthesized, and so less GlcCer would need to be catabolized in the lysosome. In other words, in a disease resulting from defective GlcCer

catabolism, if the cell makes less GlcCer, synthesis and catabolism are potentially re-balanced. Despite Radin's strong advocacy for this approach over many years [24] and his synthesis of the PDMP series of potent GCS inhibitors [25], the pharmaceutical industry did not embrace this concept. The main concern was whether inhibition of GSL biosynthesis would be tolerated to make this a viable clinical approach, although studies in animal models argued against this concern [26].

The reduction of this approach to practice had to wait almost 20 years with the serendipitous discovery of another class of GCS inhibitors. These new inhibitors were surprising as they were imino sugar drugs known previously as inhibitors of an *N*-glycan processing enzyme (alpha-glucosidase I and II). The prototypic drug was the glucose mimetic *N*-butyldeoxynorimycin, which later became known as miglustat when developed for clinical use [27,28]. Miglustat also inhibits the non-lysosomal GCase encoded by *GBA2* potently and is a less potent inhibitor of the lysosomal GCase, *GBA1* [29]. What proved to be crucial for the ability of miglustat to inhibit GCS was a minimal alky chain length of four carbons that conferred some molecular similarity to ceramide, which could potentially explain its ability to inhibit CGS. Miglustat was less potent than Norman Radin's ceramide mimetic compounds (the PDMP series) but had the translational advantage of having been scaled up and moved into clinical trials [30]. Indeed, miglustat was first trialed in man in HIV patients, as the drug was an inhibitor of HIV replication *in vitro* based on its inhibition of the N-glycan processing enzymes, alpha-glucosidase I and II [30]. Miglustat was therefore relatively straightforward to repurpose and was the first oral, CNS penetrant drug taken into clinical trials for a LSD [31].

One of the dilemmas was which GSL storage disease to trial miglustat in, as potentially multiple diseases could be treated by such an inhibitor. For example, all the LSDs with primary or secondary storage of GSLs (e.g. Tay-Sachs, Sandhoff, Fabry, Gaucher, GM1

gangliosidosis and Niemann-Pick type C) could in theory benefit from this approach. Again Roscoe Brady's pioneering research into Gaucher disease became an important factor. As type 1 Gaucher disease had clear, well defined clinical endpoints and the highly effective ERT developed by Brady as a comparator, the decision was made by Oxford GlycoSciences, who developed the drug, to move miglustat into clinical trials in type 1 Gaucher disease. If successful, the expectation was that it would add to patient choice by offering an oral alternative to intravenous ERT. Miglustat was therefore trialed over 12 months in type 1 Gaucher disease patients, measuring liver and spleen volume and hematological parameters and efficacy was demonstrated [31]. Miglustat was subsequently approved by the EMA and FDA as a second line drug if patients were unsuited to ERT. Miglustat has been reported to chaperone certain Gaucher disease mutant forms of GCase [32]so the possibility cannot be excluded that in certain patients, some of its clinical efficacy might be contributed by its chaperone activity [11].

The main side effect of miglustat treatment is osmotic diarrhea, which often resolves in patients over time [31]. This is well-documented and known to result from inhibition of the gastrointestinal disaccharidases [33]. A second imino sugar, *N*-butyldeoxygalactonjirimycin (the galactose analogue of miglustat, now called lucerastat), avoids this side effect and is currently in Phase Ib trials in Fabry disease (Actelion, Basel, Switzerland).

After the development of miglustat for SRT and positive clinical trial outcome data, there was renewed interest in the SRT approach. Hans Aerts and colleagues developed another class of imino sugar drugs that also inhibit GCS and could be used to inhibit GSL biosynthesis *in vitro* and *in vitro* [34]. Jim Shayman, who was a close colleague of Norman Radin, continued to work on the development of the PDMP series of compounds [35]. Genzyme then developed a translatable analogue derived from the ceramide mimetics that Radin and Shayman had developed, leading to the oral drug eliglustat that was trialed in type

1 Gaucher disease, resulting in FDA and EMA approval [36]. One complication of eliglustat is that patients have to be genotyped to determine their CYP2D6 metabolism status before taking the drug, and in patients with certain forms of heart disease (arrhythmias and long Qt) this drug is contraindicated [37]. When recently compared, eliglustat had a greater impact on biomarkers in Gaucher disease than miglustat, which is consistent with the differential potencies of these two drugs [38].

To date, SRT has also been approved for treating CNS manifestations in another LSD. The disease in question is the neurodegenerative LSD, Niemann-Pick type C disease, the progression of which is significantly slowed by treatment with miglustat [39]. Miglustat is approved in most countries worldwide for treating the neurological manifestations of NPC disease, but not by the FDA. Miglustat has been shown to be the first specific disease modifier of NPC that extends life in patients by improving swallowing, thereby reducing episodes of aspiration pneumonia, a common cause of death in advanced NPC disease. In the future it will be interesting to see if second generation SRT compounds [33] with greater CNS penetrance can show greater efficacy in neurodegenerative GSL storage diseases based on promising animal model studies with miglustat and lucerastat.

At the time of writing, Gaucher disease, this rare LSD synonymous with Roscoe Brady, has three approved ERT treatments and two oral SRT drugs on the market. This is a remarkable reflection of Roscoe's pioneering science and translational medicine.

Closing comments

In the current brief review, we have discussed how Dr. Roscoe Brady's seminal work shaped subsequent studies on the use of GCase in ERT and paved the way for inhibiting GlcCer synthesis with SRT. However, Dr. Brady also recognized that additional therapeutic modalities could be extremely useful in treating Gaucher disease patients, hence his involvement [40] in developing potential chaperones to rescue misfolded glucocerebrosidase.

However, we would like to conclude by suggesting that even more additional approaches are needed and there is no doubt that delineating the pathological pathways involved in Gaucher disease [15], and indeed in other LSDs, is likely to lead to additional novel therapeutic approaches in the future.

Acknowledgments

A.H. Futerman is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science. F. M. Platt is a Royal Society Wolfson Merit Award Holder and Wellcome Trust Investigator in Science.

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Figures

Fig. 1. Some key dates in Gaucher disease research.

1882:	Gaucher disease (GD) (type 1) discovered by Philippe Gaucher
1895:	A 6-year-old patient was identified suggesting that the age at which Gaucher disease manifested itself varies considerably
1901:	Brill suggested that GD is a familial disorder
1924:	Lieb suggested that cerebroside is the accumulating material
1927:	Neurological impairment first reported (now known as the infantile form – type 2)
1934:	Aghion reported that cerebroside contains glucose rather than galactose
1950s:	de Duve discovered the lysosome
1959:	Juvenile neurological form reported (type 3)
1965:	Brady identified the enzyme responsible for cerebroside metabolism
1967:	Enzyme assay first performed in white blood cells from Gaucher patients
1974:	First proof of principle of enzyme replacement therapy (ERT) by Brady
1985:	Glucocerebrosidase gene identified
1994:	First ERT approved for type 1 GD patients
2002:	Substrate reduction therapy (SRT) first approved
2003:	Glucocerebrosidase crystalized
2006:	First viable mouse model generated which mimicked GD
2009:	Genetic connection with Parkinson's disease first suggested

Fig. 2. The 3D structure of GCase. All three domains are shown (domain I: light pink; domain II: light blue; domain III: green). Catalytic residues are colored cyan. Mutants that cause severe phenotype are colored red, mild phenotype-causing mutants are colored yellow and mutants causing unknown phenotypes are colored blue.

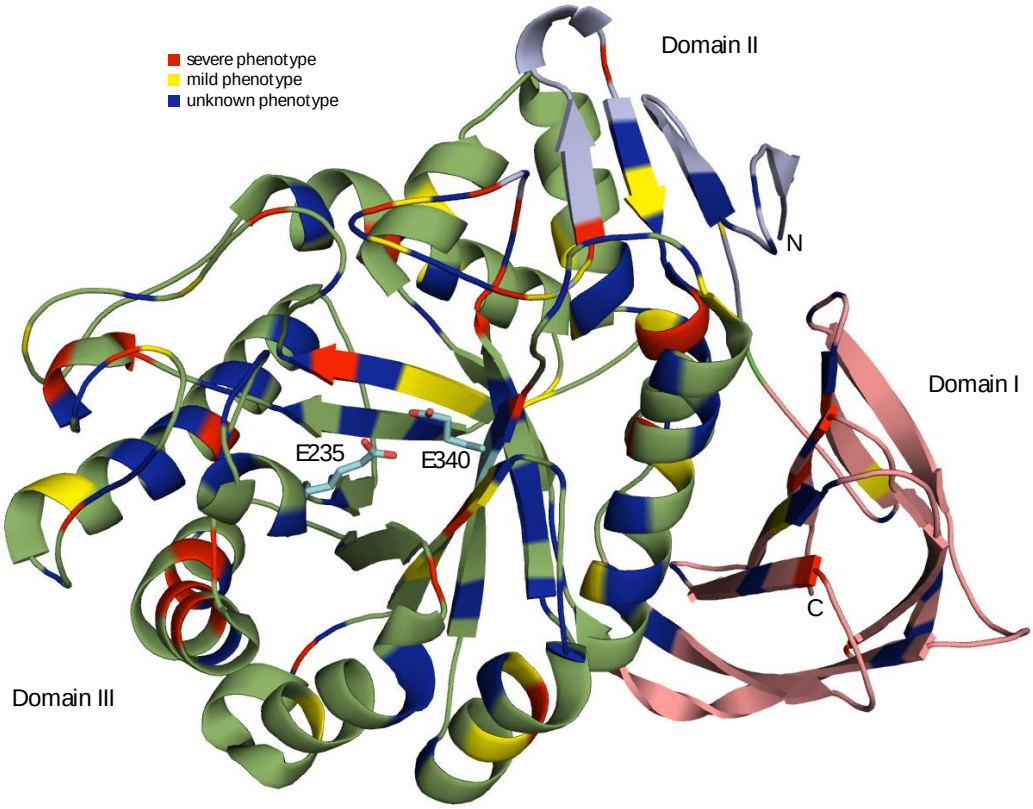

Fig. 3. The concepts of ERT and SRT. Intracellular localisation of GlcCer synthesis (early Golgi apparatus) catalysed by GCS and the target of SRT. GlcCer catabolism occurs primarily in the lysosome by the action of GCase and is the site of action of ERT.