

Review article

Carbohydrate- and heterocycle-based β -glucocerebrosidase inhibitors: State of the art and emerging relationship with cancer treatment

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ARTICLE INFO

Keywords:

β -glucocerebrosidase
GCCase
GBA1
Cancer
Overexpression
Inhibitors
Iminosugar

ABSTRACT

Overcoming drug resistance remains a significant challenge in cancer treatment. The enzyme β -glucocerebrosidase (GCCase) plays several roles in cellular processes, including carbohydrate metabolism and the hydrolysis of glucosides. Aberrant GCCase activity has been linked to various diseases, and recent studies have implicated it in cancer, where increased levels of GCCase are frequently observed. This Review examines the relationship between GCCase and cancer, emphasizing its potential as a therapeutic target. Inhibitors with diverse chemical structures, ranging from carbohydrate-based to non-carbohydrate-based compounds, exhibit varying levels of specificity for GCCase over other glycosidases. Understanding the selectivity of these inhibitors is crucial for advancing therapeutic strategies. By examining the complex interplay between GCCase, cancer, and inhibitor development, this Review aims to provide a foundation for future research and innovative therapeutic approaches in oncology.

1. Introduction

Dysregulated sphingolipid metabolism is a common feature in human cancers [1–5]. Sphingolipids are essential for cellular structure and lipid bilayer fluidity, while also functioning as bioactive signaling molecules [6,7]. These metabolites regulate critical processes, such as cell growth, differentiation, apoptosis, and autophagy, by modulating receptors, protein kinases, phosphatases, and ion transporters [8]. The sphingolipid metabolic pathway is crucial for cellular function, with ceramide being a central molecule in both biosynthesis and catabolism [9–12]. Among its derivatives, ceramide 1-phosphate (C1P) has emerged as a key player in cancer biology due to its involvement in various cellular processes that are crucial for tumor growth, survival, and metastasis [13–16]. Ceramide is produced either through *de novo* synthesis or the hydrolysis of complex sphingolipids, a process regulated by lysosomal or non-lysosomal degradation (Fig. 1). Specific β -glucosidases and galactosidases then hydrolyze these lipids to produce ceramide, which can be further deacylated by acid ceramidase to form sphingosine [17].

The *GBA1* gene encodes the lysosomal enzyme β -glucocerebrosidase (also known as GCCase, as we refer to it in this Review, EC 3.2.1.45, OMIM*606463, GBA1, or acid β -glucosidase), which hydrolyses the glycolipid substrates glucosylceramide (GlcCer) and glucosyl-sphingosine (Lyso-Gb1) into glucose and ceramide or glucose and sphingosine, respectively [18]. Genetic deficiency of GCCase leads to the accumulation of these glycolipid substrates, causing the lysosomal storage disorder known as Gaucher disease [19,20]. Moreover, mutations in *GBA1* are the most common genetic risk factor for Parkinson's disease and dementia with Lewy bodies (DLB) [21–23]. More recently, GCCase has been also implicated in triggering a form of chemoresistance to common chemotherapeutics [24–27], prompting researchers to investigate the connection of this enzyme with cancer. Indeed, its inhibition may synergistically enhance the efficacy of anti-tumor drugs.

In particular, accumulating evidence published between January 2009 and March 2026 has revealed that altered GCCase expression is implicated in the development and progression of several malignancies, including breast, ovarian, hepatocellular, and gastric cancers (see Section 1.1). While this work was in progress, a perspective highlighted the

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emerging role of the *GBA1* gene in cancer by integrating evidence from the literature with bioinformatic analyses of publicly available cancer databases, suggesting that germline mutations or somatic alterations may influence tumorigenesis and therapeutic response [28].

Moreover, both genetic depletion and pharmacological inhibition of GCase have been shown to enhance the sensitivity of cancer cells to chemotherapeutic agents *in vitro* and *in vivo*, highlighting GCase as a potential therapeutic target and providing the rationale for the pharmacological strategies discussed in this Review (see Section 1.2).

1.1. Aberrant activation of GCase and chemoresistance

Over the past decade, evidence has emerged linking the overexpression of GCase to cancer and decreased chemotherapy efficacy. Zhou et al. [24] conducted a comparative analysis of *GBA1* transcript levels, expression, and GCase activity in both normal human breast cell lines and malignant counterparts, finding significantly elevated *GBA1* mRNA expression and GCase activity in breast cancer cell lines compared to normal breast cells. They also confirmed increased GCase levels in tumor breast tissues from stage IV breast cancer patients [24]. To understand the role of GCase in breast cancer, a *GBA1* overexpression study using BglB/pET29b(+) (plasmid encoding the bacterial β -glucosidase BglB) in two normal mammary cell lines was performed, leading to increased GCase activity correlated with elevated cell proliferation and phosphorylation of the PI3K/Akt/mTOR (PAM) pathway. This highly conserved signal transduction network plays a crucial role in regulating essential cellular processes, including cell survival, growth, and cell cycle progression [29], and its dysregulation has been widely reported in various malignancies, serving as a key driver in cancer [30, 31].

Recently, Wang et al. [25] observed a notable increase in *GBA1* expression in ovarian cancer compared to normal cells using a web server for cancer and normal gene expression profiling (Gene Expression Profiling Interactive Analysis, RNA-Seq GEPIA), validated by immunohistochemistry (IHC), Western blot, and ELISA assays. *GBA1* overexpression in ovarian cancer cells increased proliferation and decreased

apoptosis post-cisplatin treatment, indicating that *GBA1* overexpression diminishes the cytotoxic effects of anti-tumoral agents [25].

High levels of GCase expression have also been observed in prostate, colon, and glioblastoma cancer cell lines at different stages of tumor progression [32,33]. Arafa et al. found varying GCase activity in Caco-2, HT29, and T84 colon cancer cell lines [33]. Specifically, the enzyme activity in Caco-2 cells was 124% and 229% higher than that in HT29 and T84 cells, respectively. Additionally, GCase activity in HT29 cells exceeded that in T84 cells by 46%. The cytotoxicity of glufosfamide (the first cytotoxic glycoconjugate synthesized by conjugating glucose with ifosfamide mustard [34], Fig. 2) in these cell lines varied with enzyme expression levels, demonstrating a concentration-dependent effect.

Zhang et al. [26] investigated GCase as a biomarker in hepatocellular carcinoma (HCC). Their immunohistochemical analysis revealed high cytoplasmic staining of GCase in advanced tumor liver tissues (patients with HCC stage 3 onwards), contrasting with low or undetectable staining in adjacent normal liver tissues. Western blot analysis confirmed an approximately 8-fold increase in GCase levels in HCC compared to normal liver tissue. Chen et al. [35] reported similar findings, with significantly higher *GBA1* mRNA levels in human HCC compared to non-tumor liver tissues, as determined by microarray analysis and real-time quantitative PCR. Consistent with patient sample data, GCase activity in several HCC cell lines was 6-fold higher than in normal liver cells [26]. Elevated GCase expression was particularly notable in poorly and moderately differentiated HCC cells compared to well-differentiated ones, indicating a potential correlation between GCase expression and disease progression. Furthermore, treatment of HCC cell lines, expressing either high or low levels of GCase, with conventional chemotherapy drugs (doxorubicin, DOX, Fig. 2 and 5-fluorouracil, 5-FU, Fig. 2) revealed a more pronounced inhibitory effect on cell proliferation when GCase expression was low, suggesting increased chemotherapy resistance in HCC cells with high GCase levels [26]. Li et al. explored chemotherapy-related resistance in gastric cancer cells [27], revealing a time-dependent upregulation of *GBA1* expression and GCase activity upon prolonged exposure to 5-FU. IHC analysis of GCase in gastric cancer tissues pre- and post-chemotherapy (5-FU with

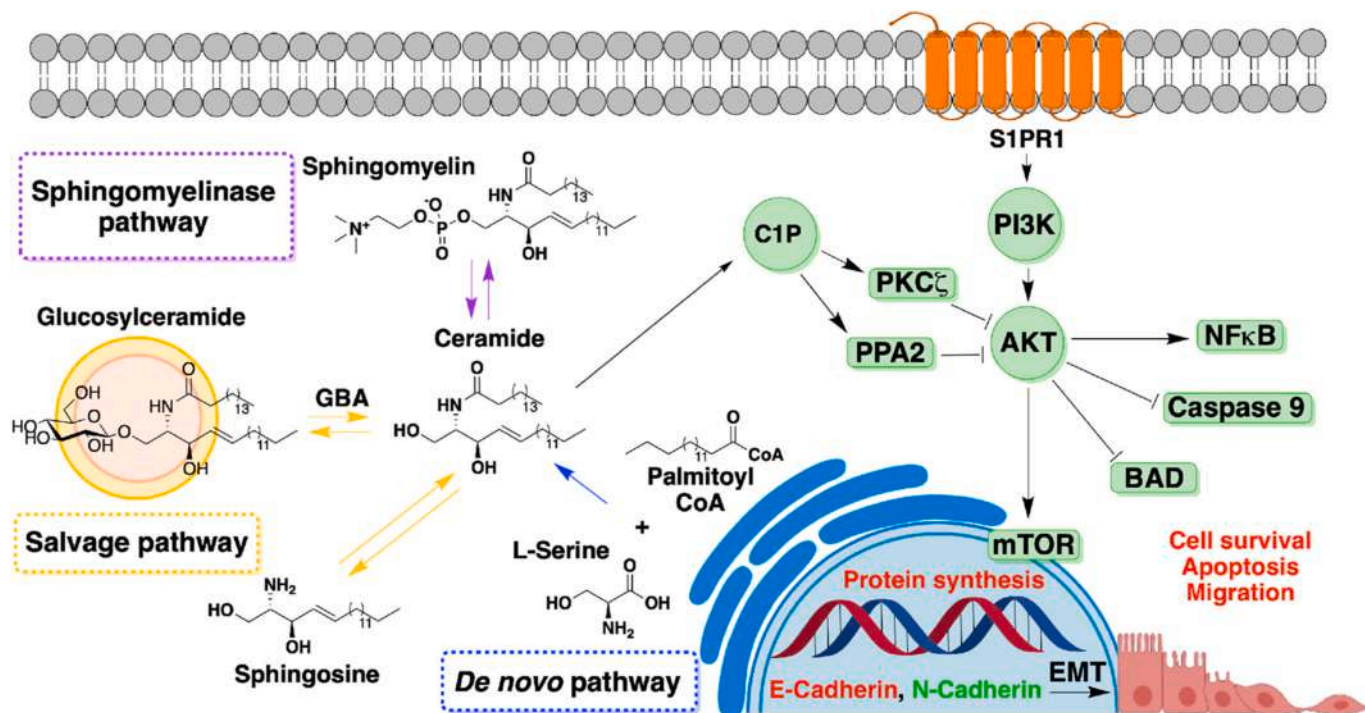


Fig. 1. Schematic illustration of the ceramide synthesis through three main pathways: *de novo* pathway, salvage pathway, and sphingomyelinase pathway, highlighting its role in cellular signaling processes that regulate cell proliferation, apoptosis, and migration.

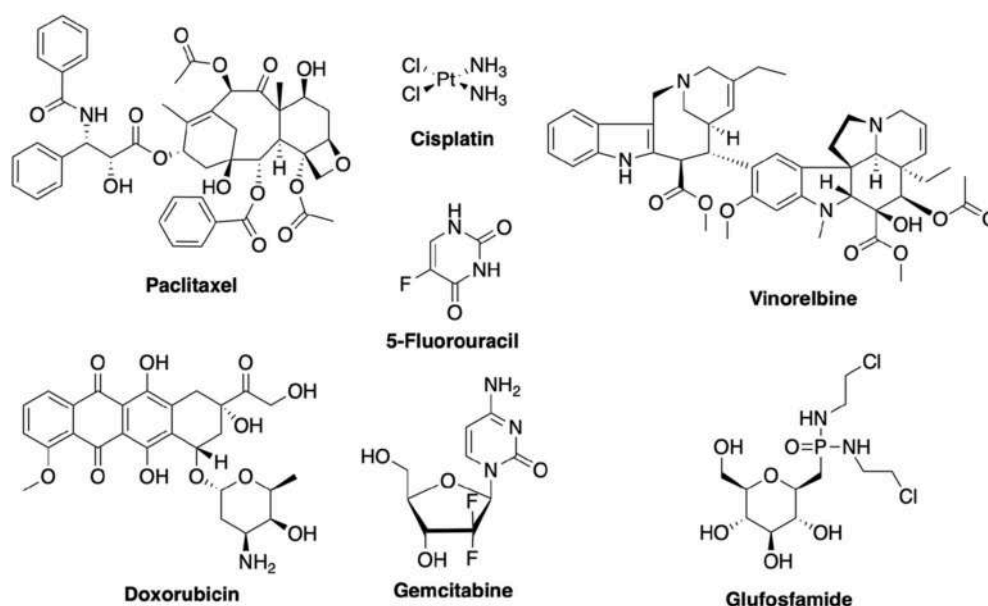


Fig. 2. Chemical structure of some commonly used chemotherapeutic agents.

cisplatin and/or paclitaxel, PTX, Fig. 2) demonstrated a consistent pattern in the response of gastric cancer to chemotherapy. Both *in vitro* and *in vivo* models suggested that increased GCase expression is linked to gastric cancer chemoresistance [27]. Despite the overall increase in GCase levels post-chemotherapy in gastric tumor tissues, no significant differences were observed between pre-chemotherapy tumour tissues and healthy tissues.

Collectively, these studies conclude that elevated *GBA1* expression and GCase activity are consistently associated with various cancers (Table 1), suggesting a potential role in increased proliferation and chemoresistance, and warranting further exploration of targeted therapeutic interventions.

1.2. Strategies to enhance chemotherapy sensitivity through GCase inhibition

To confirm the role of GCase in breast cancer progression and response to chemotherapy, Zhou et al. investigated breast cancer cell

proliferation and apoptosis by pharmacologically or genetically inhibiting GCase in combination with a chemotherapeutic agent [24]. Treatment with PTX alone did not affect GCase activity levels. Conversely, genetically engineered enzyme inhibition not only drastically inhibited proliferation and triggered apoptosis in breast cancer cells but also enhanced the anti-proliferative and pro-apoptotic effects of the chemotherapeutic agent. With respect to pharmacological inhibition, treatment with the selective irreversible GCase inhibitor conduritol β -epoxide (CBE) resulted in a significant dose-dependent inhibition of proliferation and induction of apoptosis in MDA-MB-231 and SkBr-3 cells. The combination of CBE with PTX exhibited a more substantial combined effect on cancer cells than either the chemical inhibitor or chemotherapy agent alone. In the same work, the *in vivo* efficacy of GCase inhibition in combination with PTX in a mouse xenograft model of breast cancer was further tested. Consistent with the *in vitro* data, the combination of PTX with CBE demonstrated significantly higher efficacy in arresting tumor growth compared to single-agent treatment [24].

In ovarian cancer, silencing *GBA1* using two siRNA in SK-OV-3 and Caov3 cells resulted in a 40-50% growth inhibition and 30-40% apoptosis compared to the control group [25]. Notably, *GBA1* depletion, when combined with cisplatin, led to a significant reduction in growth and an increased induction of apoptosis, exceeding the effects observed with either cisplatin or *GBA1* inhibition alone. These findings highlight the efficacy of GCase inhibition against ovarian cancer and its ability to enhance the cytotoxic effects of cisplatin. To understand the molecular signaling pathways associated with *GBA1* depletion, the authors conducted immunoblot analysis of molecules involved in ovarian cancer growth and survival. Their findings align with those observed by Zhou et al. in breast cancer [24], demonstrating that *GBA1* depletion leads to reduced expression levels of the receptor tyrosine kinase AXL (RTK-AXL) signaling pathway. This pathway is implicated in various aspects of tumor cell biology, including growth, metastasis, invasion, epithelial-mesenchymal transition (EMT), angiogenesis, drug resistance, immune regulation, and stem cell maintenance, with interruption of the signaling cascade [37–39]. Conversely, no differences in RTK-AXL and phospho-AXL (p-AXL) levels were observed between cisplatin-treated and control ovarian cancer cells, emphasizing the specific impact of *GBA1* depletion on AXL signaling.

Li et al. [27] investigated the impact of *GBA1* silencing in 5-FU resistant gastric cancer cells, revealing a significant decrease in

Table 1
Overview of *GBA1* gene or GCase enzyme overexpression in various tumor types.

Organ systems	Finding(s)	Ref(s)
Reproductive	Elevated <i>GBA1</i> mRNA, GCase activity, and expression in breast cancer cell lines. Increased cell proliferation and activation of the PI3K/Akt/mTOR pathway with GCase overexpression.	[24, 36]
Reproductive	Significantly higher GCase expression in tumor tissues. <i>GBA1</i> overexpression leads to increased proliferation and decreased apoptosis in response to cisplatin.	[25]
Digestive	Significant differences in GCase activity among colon cancer cell lines. Caco-2 cells show higher activity compared to HT29 and T84 cells.	[33]
Digestive	Increased GCase activity in gastric cancer cells post-chemotherapy. Consistent pattern in the response of gastric cancer to chemotherapy. Increased chemoresistance with higher level of GCase.	[27]
Hepatic	High cytoplasmic staining of GCase in advanced HCC tissues. Approximately 8-fold increase in GCase levels in HCC compared to normal liver tissue. Elevated GCase expression and activity in HCC cell lines, particularly in poorly and moderately differentiated cells. Increased resistance to chemotherapy in HCC cells with high GCase levels.	[26, 35]

protein levels and enzyme activity. This reduction correlated with decreased proliferation and increased apoptosis, while cell migration was unaffected. The study highlighted that GCCase inhibition not only enhances the anti-proliferative and pro-apoptotic effects of 5-FU in gastric cancer cells, but also results in the loss of lysosomal integrity, as evidenced by a significant reduction in α -N-acetylglucosaminidase activity and an increase in malondialdehyde levels.

Similarly, in the study by Zhang et al. [26], *GBA1* silencing demonstrated more efficient reduction in cell proliferation and increased apoptosis in HCC cells exhibiting high GCCase expression. This result was also confirmed by Chen et al. [35] using CBE as a selective and irreversible GCCase inhibitor. Moreover, combining CBE with chemotherapeutic agents (DOX or 5-FU) proved to be a more efficacious strategy than using these compounds alone.

In a recent investigation by Clark et al. [40], the impact of *GBA1* silencing on various cancer-associated phenotypic properties was assessed. *GBA1* knockout induced the accumulation of lactosylceramides (LacCer) in HeLa cells and a significant increase in monohexosylceramides (monoHex-Cer) in H1703 cells. The genetic reduction of *GBA1* expression led to a mesenchymal-epithelial transition (MET), decreased invasion and migration, increased sensitivity to chemotherapy, and decreased activation of receptor tyrosine kinases involved in regulating EMT. However, pharmacological modulation of GCCase with CBE did not induce the MET phenotypic changes resulting from *GBA1* gene deletion [40]. Similarly, the use of an inhibitor targeting GlcCer synthase, which reduces the availability of the substrate cleaved by GCCase, did not result in observable changes. The study suggested that the relationship between GlcCer levels and chemoresistance may depend on the extent of cell dependence on GCCase activity to maintain EMT. The simultaneous inhibition of GCCase and GlcCer synthase has been proposed as a potentially effective therapeutic strategy in selected cancer types, as dual targeting of glycosphingolipid metabolism may help overcome both chemoresistance and metastatic progression. However, this therapeutic concept remains largely unexplored and is currently supported by limited experimental evidence [41–43]. Because GCCase modulation profoundly affects glycosphingolipid metabolism, the selectivity of GCCase inhibitors, particularly their off-target activity toward GlcCer synthase, should be carefully evaluated to maximize therapeutic benefit while minimizing adverse effects. When developing GCCase inhibitors as chemosensitizing agents in combination therapies, systematic assessment of GlcCer synthase cross-reactivity is essential to achieve an optimal balance between efficacy and safety. From a translational perspective, the availability of clinically approved GlcCer synthase inhibitors (e.g., miglustat and eliglustat) offers a valuable opportunity to explore combination or sequential treatment regimens aimed at fine-tuning glycosphingolipid metabolism while mitigating the potential toxicity of sustained GCCase inhibition. However, CBE failed to replicate the effect of *GBA1* depletion on EMT, possibly due to its inhibition of multiple β -glycosidases, preventing the accumulation of specific glycosphingolipids (GSLs) mediating this effect [40]. In a study by Yanagaki et al. [44], *GBA1* knockdown demonstrated a suppressive influence on proliferation across all pancreatic cell lines. Western blot and flow cytometry analysis revealed heightened apoptotic protein expression, indicating increased apoptotic signals. Notably, the intense anti-proliferative effects correlated with significant alterations in intracellular organelles, particularly in mitochondria, which exhibited accumulation, swelling, and the generation of reactive oxygen species (ROS). These findings suggest that ROS generation on accumulated mitochondria induces damage and triggers mitochondria-derived apoptosis signals. Consequently, *GBA1* knockdown contributes to mitochondrial dysfunction, apoptosis, and the inhibition of proliferation in malignant tumor cells. Furthermore, the combination of *GBA1* knockdown with anticancer drugs (e.g., gemcitabine (GEM, Fig. 2) and 5-FU) resulted in increased antiproliferative and cell apoptosis effects compared to gemcitabine or 5-FU alone [44].

The high expression of GCCase observed in certain tumor cell lines

also presents an opportunity to exploit this enzymatic activity for therapeutic purposes. By designing prodrugs or compounds specifically cleaved by GCCase, a cytotoxic aglycone can be selectively released within the tumor microenvironment. Examples of therapeutic exploitation include the use of synthetic or natural glycoconjugates, such as glufosfamide and amygdalin. Following GCCase-mediated hydrolysis of the glycosidic bond, cytotoxic compounds like ifosfamide mustard and hydrocyanic acid are released, exerting toxicity toward cancer cells [32, 36,45–47].

In conclusion, various studies consistently highlight a significant role of GCCase in cancer progression and response to chemotherapy across different cancer types (Table 2). Overall, these studies collectively underscore the potential of targeting GCCase and related pathways as a promising avenue for cancer therapy, particularly when combined with standard chemotherapeutic agents to achieve a greater combined effect. Furthermore, the ability of GCCase to act as a biological trigger for prodrugs further emphasizes its versatility in cancer-targeted therapy. Using the elevated activity of GCCase in tumors presents a valuable opportunity to develop innovative therapeutic approaches, potentially unveiling new avenues in cancer treatment research.

1.3. Structural and mechanistic basis of GCCase function: implications for selective inhibitor design

GCCase is a peripheral membrane protein composed of 497 amino acids, with a molecular weight ranging from 59 to 69 kDa, depending on post-translational modifications [48]. GCCase is structured into three discontinuous domains: (i) Domain I, consisting of residues 1–27 and 383–414, features an antiparallel β -sheet flanked by a loop. Within this domain, there are two disulfide bridges (residues 4–16 and 18–23) that likely facilitate proper protein folding; (ii) Domain II (residues 30–75 and 431–497), comprising two associated β -sheets that form an independent domain resembling an immunoglobulin (Ig) fold; (iii) Domain III (residues 76–381 and 416–430), containing the active site and three cysteine residues (at positions 126, 248, and 342) [49].

GCCase belongs to the family of retaining glycosidases, which operate through a two-step reaction mechanism. Each step involves an inversion, resulting in a net retention of stereochemistry at the sugar anomeric carbon (Fig. 3). This process involves the glucosylation of the active site by the substrate, followed by hydrolytic deglucosylation with the release of β -glucose [50]. The reaction is facilitated by acid/base catalysis. In the active site, Glu340 acts as the nucleophile [51], while Glu235 serves as the acid/base catalyst [52]. The O-glucoside is protonated by the acid/base catalyst (Glu235), while the anomeric carbon is attacked by the nucleophile (Glu340) (Fig. 3). This results in the covalent bonding of glucose to the protein and the departure of the leaving group. The nucleophilic attack of a water molecule, facilitated again by the acid/base catalyst, leads to the release of β -glucose, thus retaining the original anomeric configuration.

In addition to lysosomal GCCase, two other enzymes catalyze the hydrolysis of GlcCer to glucose and ceramide, namely bile acid β -glucosidase (GBA2, OMIM*609471) and cytosolic β -glucosidase (GBA3, OMIM*606619). Like lysosomal GCCase, both enzymes catalyze GlcCer hydrolysis through the same retaining, double-displacement mechanism. Although the three enzymes share similar enzymatic activity, they lack structural or sequence homology [53–55], belonging to different Carbohydrate-Active Enzymes (CAZy) glycoside hydrolase (GH) families and differing substantially in overall architecture (Table 3).

GCCase (GBA1) belongs to family GH30, clan GH-A, and its catalytic domain (Domain III) is a $(\beta/\alpha)_8$ TIM-barrel bearing the catalytic dyad Glu235 (acid/base) and Glu340 (nucleophile) [49]. GBA3 also adopts a clan GH-A $(\beta/\alpha)_8$ TIM-barrel fold, despite belonging to the distinct family GH1, with an equivalent Glu-Glu catalytic dyad (Glu165 acid/base, Glu373 nucleophile) positioned at the C-terminal ends of barrel strands 4 and 7 [58]. GBA1 and GBA3 therefore share the same overall

Table 2

Summary of the impact of genetic and pharmacological GCase inhibition in combination with anticancer agents.

Cancer type	Inhibition method(s)	Combination therapy	Outcome(s)	Ref (s)
Breast	RNA interference and competitive Inhibition	PTX and CBE	Genetically engineered enzyme inhibition significantly inhibited proliferation and induced apoptosis in breast cancer cells. Combination of CBE with PTX showed greater anti-proliferative and pro-apoptotic effects compared to individual treatments.	[24]
Ovarian	RNA interference	siRNA and Cisplatin	<i>GBA1</i> knockdown significantly enhanced anti-proliferative and pro-apoptotic effects of the antitumoral agent. The loss-of-function approach of the <i>GBA1</i> gene induces a partial reduction in the expression of the AXL signaling pathway.	[25]
Gastric	RNA interference	siRNA and 5-FU	Cell migration remained unaffected by <i>GBA1</i> silencing. <i>GBA1</i> inhibition enhanced the anti-proliferative and pro-apoptotic effects of 5-FU in gastric cancer cells and caused a loss of lysosomal integrity.	[27]
Hepatocellular	RNA interference	DOX or 5-FU and CBE	Silencing of <i>GBA1</i> showed heightened efficacy in decreasing cell proliferation and promoting increased apoptosis. Combining CBE with DOX or 5-FU proved more effective than using them alone.	[26, 35]
Cervical/Lung	RNA interference and competitive inhibition	PTX/DOX/ Cisplatin/ Vinorelbine and CBE or siRNA	<i>GBA1</i> knockout induced the accumulation of LacCer in HeLa cells and a significant increase in monoHex-Cer in H1703 cells. Pharmacological modulation of GCase did not induce phenotypic changes in MET.	[40]
Pancreatic	RNA interference	siRNA and GEM or 5-FU	Silencing of <i>GBA1</i> induces an increase of apoptotic signals and alteration of mitochondria.	[44]

Table 2 (continued)

Cancer type	Inhibition method(s)	Combination therapy	Outcome(s)	Ref (s)
			Combination of silencing of <i>GBA1</i> with antitumor agents showed increased antiproliferative and proapoptotic effects.	

clan-level fold and catalytic geometry despite very low pairwise sequence identity, which helps explain why some inhibitors (e.g., CBE) show only modest discrimination between the two in certain assay systems.

In contrast, GBA2 belongs to the structurally unrelated family GH116 and is built from an N-terminal β -sandwich (immunoglobulin-like) domain fused to a C-terminal all- α , (α/α)₆-solenoid catalytic domain, a fold with no relationship to the TIM barrels of GBA1/GBA3 [59]. No experimental structure of human GBA2 has yet been reported; therefore, current structural knowledge relies on its bacterial homolog *Thermoanaerobacterium xylanolyticum* GH116 (TxGH116), which shares ~32% overall sequence identity and ~40% identity within the catalytic domain, with complete conservation of the active-site residues, as well as on homology models derived from this template. In this fold, the catalytic dyad is Glu-Asp (rather than Glu-Glu), and an extended loop adjacent to the active site (longer in GBA2 than in TxGH116) is thought to shape a distinct aglycone-binding groove [59].

This divergence in fold family (GH30/GH1 TIM-barrel clan GH-A vs GH116 α -solenoid) and in the identity/geometry of the catalytic residues (Glu-Glu vs Glu-Asp) provides a structural rationale for why chemically distinct inhibitor scaffolds achieve markedly different selectivity profiles across the three enzymes, as discussed throughout this Review: compounds engaging the GBA1/GBA3-type TIM-barrel active site can, in principle, cross-react with either enzyme, whereas selectivity for GBA2 typically requires substituents that exploit the unique α -solenoid pocket and its extended loop, inaccessible to GBA1/GBA3-directed scaffolds.

As mentioned above, all three enzymes utilize the same retaining, double-displacement mechanism involving two highly conserved carboxylic acid residues: a catalytic nucleophile and a general acid/base catalyst [60,61]. However, their spatial positioning, pocket topography, and aglycone-binding regions differ fundamentally:

- GBA1 Active site: The catalytic machinery uses Glu340 (nucleophile) and Glu235 (acid/base). The catalytic pocket is deep, rigid, and possesses a highly hydrophobic, narrow cleft that accommodates the sphingosine and fatty acid acyl chains of ceramide.
- GBA2 Active site: Because mammalian GBA2 is highly unstable for sequence extraction and crystallization, its primary sequence motifs are mapped against the strictly conserved bacterial homolog TxGH116. The catalytic nucleophile is Glu441, which would correspond to Glu527 in human GBA2. The GBA2 active site pocket sits broad and highly accessible on the enzyme surface. It is significantly more solvent-exposed and exhibits less steric hindrance around the aglycone binding region than GBA1. Unlike GBA1, GBA2 can readily process both β -glucosidic and β -galactosidic substrates [57].
- GBA3 Active site: The acid/base catalyst is Glu165 (positioned within the classic GH1 TFNEP consensus motif); while the nucleophile is Glu373 (nested within the highly conserved I/V-E-N-G signature motif at the end of the 7th β -strand). Features a wide, shallow pocket typical of GH1 broad-specificity glycosidases, designed to accommodate a diverse array of polar or aromatic cytosolic glucosides rather than bulky sphingolipids.

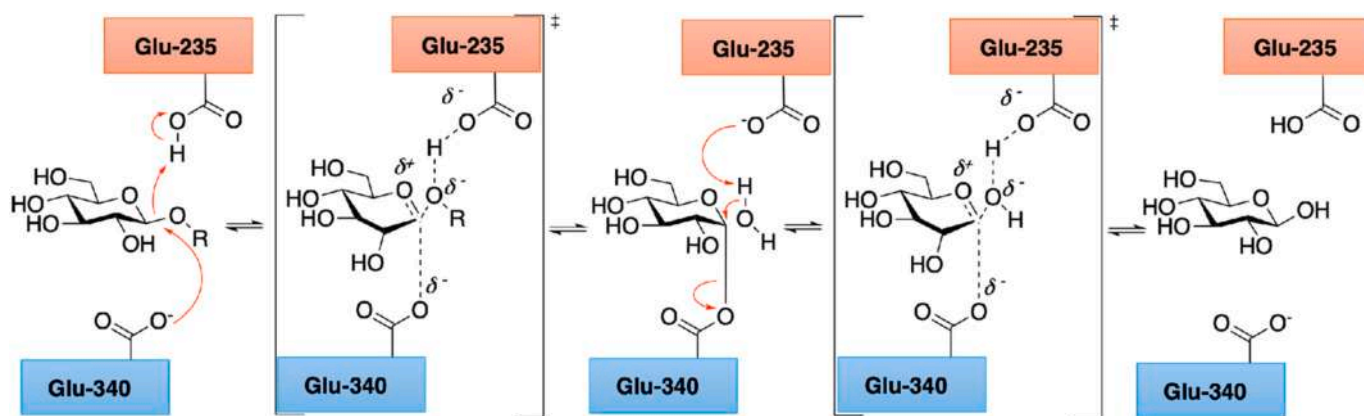


Fig. 3. Retaining, double-displacement mechanism of hydrolysis for GCase.

Table 3

Structural comparison of GCase (GBA1), GBA2, and GBA3.

Feature	GCase (GBA1)	GBA2	GBA3
CAZy family/ clan	GH30/clan GH-A	GH116/no clan assigned	GH1/clan GH-A
Gene location	Chromosome 1 (1q21)	Chromosome 9 (9p13.3)	Chromosome 4 (4p15.2)
Primary length	536 amino acids (497 in mature form)	927 amino acids	469 amino acids
Cellular localization	Lysosomal (luminal) [56]	Cytosolic-facing, membrane- associated [54]	Cytosolic (soluble) [57]
Overall fold	3 discontinuous domains; TIM- barrel catalytic domain	β -sandwich + C- terminal $(\alpha/\alpha)_6$ solenoid	Single $(\beta/\alpha)_8$ TIM barrel
Catalytic nucleophile	Glu340	Glu (homology, e. g., Glu441 in TxGH116)	Glu373
Acid/base catalyst	Glu235	Asp (homology, e. g., Asp593 in TxGH116)	Glu165
Experimental human structure	Yes (multiple, e.g., PDB 1OGS and inhibitor complexes)	No (bacterial homolog TxGH116 + homology model only)	Yes (e.g., PDB 2E9M and covalent- intermediate structure)
Relationship to GBA1 fold	—	Structurally unrelated	Same clan/fold, different family

Thus, selectivity should be obtained by exploiting differences in three-dimensional topology, pocket volume, and aglycone-channel hydrophobicity. For example, to achieve high specificity for GBA1 over GBA3 and GBA2, inhibitors must extend lipophilic motifs that specifically engage the unique hydrophobic aglycone-binding channel of the GH30 fold. This narrow channel is completely absent in the shallow pocket of GBA3 and vastly different from the broad, solvent-accessible surface of GBA2.

The present Review focuses on pharmacological strategies to inhibit GCase, emphasizing natural inhibitors and synthetic derivatives inspired by natural compounds. The most effective inhibitors generally mimic the functional and structural characteristics of the enzyme's natural substrate. We discuss compounds such as cyclitols, iminoalditols, and other sugar mimetics, as well as non-carbohydrate inhibitors, particularly highlighting those with half-maximal inhibitory concentration (IC_{50}) values up to 10 μ M to emphasize the most potent inhibitors. A key challenge in the design and development of GCase inhibitors is achieving high selectivity for lysosomal GCase (GBA1) over closely related non-lysosomal β -glucosidases (GBA2 and GBA3) and other glycosidases, including α -glucosidases, α -galactosidases, and

β -galactosidases. Accordingly, particular emphasis is placed throughout this Review on the selectivity profiles of the reported inhibitors, highlighting their specificity toward GCase relative to these off-target glycosidases.

While comparisons with human glycosidases are more relevant for therapeutic purposes, we have included data for glycosidases from other organisms when human-specific biological data is unavailable. This choice was made to provide a comprehensive overview of available knowledge, acknowledging differences between the species and limitations in direct applicability to human systems. Another important consideration in the development of GCase inhibitors is whether enzyme inhibition should be reversible or irreversible. Irreversible inhibitors often provide prolonged target engagement and high apparent potency, representing invaluable tools for activity-based protein profiling and target validation. On the other hand, reversible inhibitors offer greater control over enzyme modulation and may reduce the risk associated with sustained suppression of GCase activity. Accordingly, throughout this Review, the reversible or irreversible nature of each inhibitor class is highlighted whenever this information has been reported by the original authors.

2. Enzyme activity assay for GCase

This Review will examine the efficacy and specificity of various GCase inhibitors, focusing on their potential uses and limitations. Over recent decades, significant efforts have been dedicated to designing and synthesizing GCase inhibitors. Typically, the most potent inhibitors mimic the functional and conformational characteristics of the enzyme's natural substrate [62,63]. We will explore common inhibitors based on both carbohydrate and non-carbohydrate structures, with IC_{50} values ranging from low micromolar to nanomolar, assessing their specificity towards GCase compared to non-lysosomal β -glucosidases (GBA2 and GBA3), α -glucosidase, α -galactosidase, and β -galactosidase. The selectivity of compounds toward the target glycosidase is essential to minimize off-target effects and ensure therapeutic efficacy. This specificity is particularly important in treating diseases where the dysregulation of a specific glycosidase is implicated. By designing compounds that selectively inhibit GCase, the likelihood of unwanted interactions with other glycosidases, which could lead to side effects or reduced effectiveness, is minimized.

Enzyme activity assays are commonly performed for the measurement of GCase activity, particularly in the diagnosis of Gaucher disease. Several *in vitro* techniques are available for measuring enzymatic activity, including fluorescence assays [64–66] and chromogenic assays [67,68], which utilize 4-methylumbelliferyl- β -glucopyranoside and *p*-nitrophenyl- β -glucoside, respectively, as non-physiological β -glucosidase substrates. These assays involve the enzymatic cleavage of the O-glycosidic bond, resulting in the release of an aglycone that is then

quantified using fluorometric or spectrophotometric methods. Fluorescence assays are among the most widely used techniques in diagnostics. The enzymatic activity of β -glucosidases, which hydrolyze 4-methylumbelliferyl- β -glucosidase, is typically determined using McIlvaine buffer solution containing 0.10–0.25% (v/v) Triton X-100 (TX-100) [69, 70] and 0.1% (w/v) Bovine Serum Albumin (BSA) [71]. These assays are typically performed at optimal pH values, usually at 37 °C for durations ranging from 10 to 180 min, with or without pre-incubation at 0 °C or 37 °C for 30–45 min [72,73]. After incubation with the inhibitor and enzyme, the reaction is typically stopped using a basic solution, such as sodium carbonate at pH 10.7 [69,74].

Discrimination between GCase, GBA2, and GBA3 isoforms in enzymatic assays relies on using the same substrate but varying the pH, as each isoform has a distinct pH optimum. This method enables clear differentiation of their enzymatic activities, reflecting their specific functions in different cellular compartments and under unique chemical environments [56,75]. In particular, the use of sodium taurocholate detergent is crucial in GCase assays, serving as an essential component that allows for the accurate measurement of GCase activity when using artificial substrates [64,76], while reducing interference from other enzymes that exhibit glucosidase activity [77,78]. Indeed, sodium taurocholate selectively inhibits non-lysosomal β -glucosidase activity and is typically used in a McIlvaine buffer at 0.20–0.30% (w/v) [71,74, 79].

To better evaluate selectivity, additional inhibitors of β -glucosidases, other than GCase, are commonly included in the buffer solution or pre-incubated before the assay is performed. For instance, to exclude GBA2 activity when testing GCase inhibition, 25 nM *N*-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM), a substrate that selectively inhibits GBA2, is used [80]. Conversely, when studying GBA2 inhibition, pre-incubation with a compound that selectively inhibits

GCase, such as CBE, is conducted [71,80–82].

Various sources are used to evaluate GCase, GBA2, and GBA3 activities. For GCase, recombinant enzymes used for Gaucher therapy (VPRIV™, Cerezyme™, Elelyso™), leukocyte extracts, HEK293T cells, and zebrafish larvae are the most common sources of enzyme (Table 4). For GBA2 and GBA3, a homogenized human splenectomised N370S/RecNcil Gaucher spleen can be used, where the supernatant serves as a source of GBA3 and the pellet, pre-incubated with CBE, is used for GBA2 [82]. Alternatively, lysates from GBA/GBA2 knockout HEK293T cells overexpressing GBA2 or GBA3, respectively, can be used [81]. Finally, HEK293T cells overexpressing GBA2, treated with GCase inhibitor, can also serve as a source of GBA2 [71].

Table 4 summarizes commonly reported assays for determining GCase, GBA2, GBA3, α -glucosidase, α -galactosidase, and β -galactosidase activities, highlighting the specific conditions employed (pH, substrate concentration, incubation time, additives, etc.) for each enzyme. Regarding GCase activity determination, slight differences in pH conditions, substrate concentration, and incubation time are observed among various research groups. However, due to the lack of ISO standards for the determination of GCase activity, we assume that the reported data can be reasonably compared for the structure-activity analysis carried out in this Review.

3. Pharmacological inhibition of GCase: carbohydrate-based inhibitors

3.1. Cyclitols

3.1.1. Conduritol β -epoxide and analogues

Among the GCase inhibitors, several carbohydrate-based structures have been synthesized and tested. Conduritol β -epoxide (1, CBE, Fig. 5),

Table 4

Experimental conditions for evaluating glycosidase activity, including buffer composition, pH level, substrate, incubation time, and additional components.

Enzyme	Buffer	pH	Substrate [mM]	Time (min)	Sodium taurocholate % (w/v)	TX-100% (v/v)	BSA % (w/v)	Ref
GCase ^a	McIlvaine	5.2	[3.0 – 4.0]	30 -180	0.20	0.10	0.10	[71,80–82]
		5.2	4MU- β -D-glc		0.25		-	[73,83]
		5.2 or 7.0					-	[70]
		5.4					-	[70,79]
GBA2 ^b	McIlvaine	5.8	[3.75]	30 - 180	0.30	0.15	-	[69,74]
			4MU- β -D-glc		-	-	-	[71,80–82]
GBA3	McIlvaine	6.0	[3.75]	30	-	-	0.10	[80,81]
α -glucosidase ^c	McIlvaine	4.0 or 7.0	[3.0]	-	-	-	0.1	[71,80]
			4MU- α -D-glc					
			[n.d.] pNP- α -D-glc					[84]
			[n.d.]	60	-	-	-	[69,74]
α -galactosidase ^e	McIlvaine ^d	5.5	4MU- α -D-glc	-	0.25	0.25	-	[84]
			DNP- α -gal					
		5.2	[3.0]	30	0.25	0.10	-	[73]
			4MU- α -D-gal					
β -galactosidase	Acetate ^f	4.5	[1.47]	60	-	-	-	[69,74]
			4MU- α -D-gal					
			[1.0]	-	-	-	-	[74]
			4MU- β -D-gal					
	Sodium phosphate	7	[n.d.] pNP- β -D-gal	-	-	-	-	[84]
		4.3	[1.47]	60	-	-	-	[69,74]
	Acetate ^g		4MU- β -D-gal					

*n.d. = not defined; 4MU- β -D-glc = 4-Methylumbelliferyl- β -D-glucopyranoside; pNP- α -D-glc = 4-Nitrophenyl- α -D-glucopyranoside; 4MU- α -D-glc = 4-Methylumbelliferyl- α -D-glucopyranoside; DNP- α -gal = 2,4-Dinitrophenyl- α -D-galactopyranoside; 4MU- α -D-gal = 4-Methylumbelliferyl- α -D-galactopyranoside; 4MU- β -D-gal = 4-Methylumbelliferyl- β -D-galactopyranoside; pNP- β -D-gal = 4-Nitrophenyl- β -D-galactopyranoside.

^a Addition of AMP-DNM or pre-incubation at 0–37 °C for 30–45 min without substrate.

^b Incubation with GBA1 inhibitors (e.g., 5 mM CBE) for 30 min at 4 °C.

^c Incubation with β -glucosidase inhibitor for 3 h at 37 °C.

^d Presence of 1.0 mM tetrasodium EDTA in buffer solution.

^e Preincubation at 0 °C for 45 min then add substrates.

^f Buffer containing sodium azide 0.02 (%).

^g Buffer solution containing 0.1 M NaCl and sodium azide 0.02 (%).

also known as 1,2-anhydro-myo-inositol, is a sugar analogue characterized by a cyclitol ring with four hydroxyl groups and a fused epoxide ring. The adjacent hydroxyl groups on the cyclitol ring introduce a significant electron-withdrawing effect, crucial for slowing down the acid-catalyzed nucleophilic substitution reaction. This stabilizes the epoxide moiety, preventing spontaneous hydrolysis before reaching the target. Conversely, CBE can undergo ring opening by a nucleophile within the GCase active site [85]. These properties enable CBE to irreversibly inhibit GCase, making it the compound of choice in studies involving GCase inactivation. CBE's inhibitory activity arises from its structural mimicry of β -D-glucopyranoside, facilitating interaction within the enzyme's active site, as confirmed by the crystal structure of human acid GCase with bound CBE [86]. Once in the active site, a nucleophilic ring opening reaction occurs, resulting in an irreversible covalent bond between CBE and the enzyme, characterized by a highly selective and specific inhibition mechanism (Fig. 4A). The inactivation process involves protonation of the oxirane oxygen by Glu235, followed

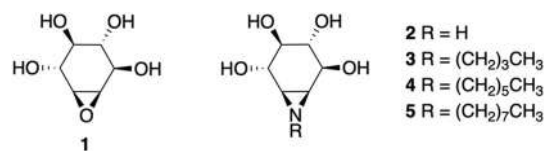


Fig. 5. Chemical structures of conduritol β -epoxide (**1**) and some conduritol aziridine analogues (**2-5**). All compounds are covalent inhibitors.

by nucleophilic ring opening with Glu340, leading to the formation of the inactive enzyme. The *trans* ring opening configuration observed in the X-ray structure of GCsase with bound CBE is particularly significant, highlighting the covalent linkage between Glu340 and the C-1 position of CBE (Fig. 4B). These findings provide valuable insights into the specific interactions involved in GCsase inactivation [86]. The absence of an endocyclic oxygen results in a highly stable covalent inhibitor-enzyme bond, which cannot be broken down by an activated water molecule.

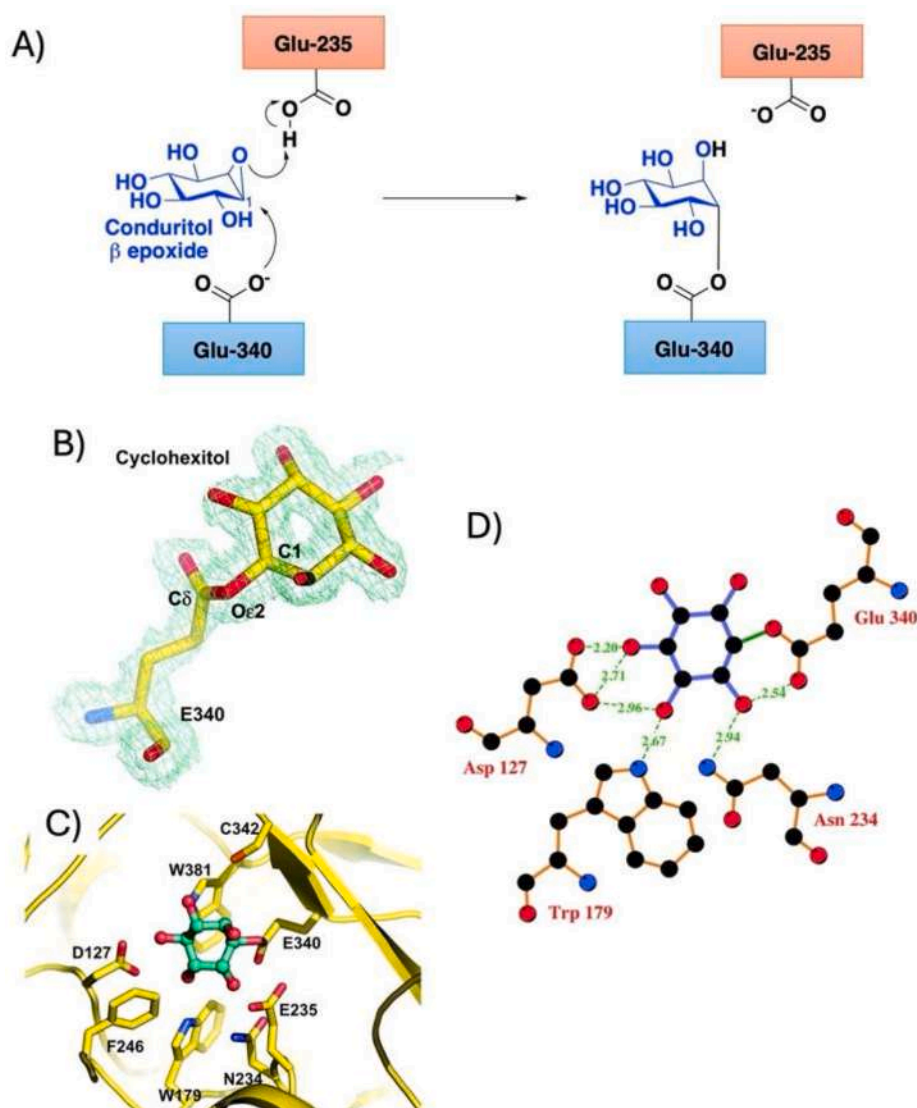


Fig. 4. A) Mechanism of GCase inhibition by CBE; Binding of CBE to the GCase active site: B) $F_o - F_c$ difference omit map (contoured to 3.2 σ level) of the cyclohexitol ring and of Glu340 in the active site of GCase; and C) active site of GCase with bound cyclohexitol. The cyclohexitol ring is in cyan, and its hydroxyl groups are in red. The catalytic nucleophile (Glu340 (E340)), the proposed acid/base catalyst (Glu235 (E235)), and the residues in proximity to CBE are displayed in CPK colors; D) Hydrogen bonding interactions of the cyclohexitol ring in the active sites of GCase with green dashed lines indicating hydrogen bonds (lengths in Å) and with the intact green line representing the covalent bond between C-1 of the cyclohexitol and the catalytic nucleophiles of the enzymes. Reprinted from Ref. [86]: Premkumar, L. et al., X-ray Structure of Human Acid- β -Glucosidase Covalently Bound to Conduritol-B-Epoxyde: **Implications for Gaucher Disease**, *J. Bior. Chem.* **2005**, *280*, 25, 23815–23819, under the terms of the Creative Commons CC-BY license.

CBE can be obtained as a racemic mixture from myo-inositol [87], as the L-enantiomer from L-quebrachitol [88,89], and as the D-enantiomer from D-glucose [90] or D-pinitol [89]. The D-enantiomer, the most active, was initially reported as an irreversible inhibitor against β -glucosidase from almond, with an IC_{50} value of 67.5 mM [89]. A more recent study, conducted *in vitro* and *in vivo*, demonstrated that the IC_{50} of CBE using recombinant human GCase (rhGCase), cultured cells, and zebrafish larvae is significantly lower, namely 4.28 μ M, 0.33 μ M, and 58.5 μ M, respectively. The selectivity of CBE for GCase, compared to GBA2, is remarkably high in cultured cells, with an 883-fold discrimination factor. In contrast, experiments conducted in fish larvae reported a selectivity for GCase only 20-fold higher compared to GBA2. When the *in vivo* targets of CBE were studied in a mouse model, GCase emerged as the primary target regardless of dosage, suggesting a therapeutic window for selective GCase inactivation by CBE. However, using CBE concentrations higher than 890 μ M or extending the incubation time beyond 5 days may lead to unwanted inhibition of other glycosidases, significantly decreasing selectivity in the zebrafish larvae model [80]. In addition, as anticipated in Section 1.2, the combination of CBE with anticancer drugs renders cancer cells more sensitive to treatment both *in vitro* and *in vivo* [24].

Conduritol aziridine (2, Fig. 5), an aza-analog of CBE with an aziridine ring replacing the epoxide, was first reported in 1989 and it was expected to exhibit similar reactivity and inactivation mechanism as CBE. The structural similarity of 2 and CBE (1) suggests that conduritol aziridine could effectively modulate GCase activity like its epoxide analogue. Notably, conduritol aziridine 2 demonstrated a higher affinity for GCase from *Agrobacterium sp.*, likely due to the positively charged nitrogen at physiological pH, which facilitates electrostatic interactions with negatively charged residues within the active site [91]. This promising result led to the introduction of hydrophobic alkyl chains of varying lengths on the aziridine core of conduritol to mimic the lipid present on the natural substrate [92]. Indeed, *N*-butyl, *N*-hexyl, and *N*-octyl analogues (3, 4, 5, Fig. 5) revealed an inverse correlation between alkyl chain length and K_i values, with measured values of 2400 nM, 50 nM, and 4.8 nM for 3, 4, and 5, respectively, suggesting effective mimicry of the natural ceramide aglycone and an increased

affinity for GCase [92]. Compound 5 emerged as the most potent inhibitor of the synthesized series, with an IC_{50} of 23 μ M at 37 °C in HeLa cells, slightly increasing to 100 μ M at 18 °C. This IC_{50} increase at lower temperature is attributed to the decrease in GCase enzymatic activity. These findings highlight the potential of *N*-alkyl-modified conduritol aziridines as irreversible GCase inhibitors, offering crucial insights into their efficacy in cellular contexts [92].

3.1.2. Cyclophellitol and analogues

Cyclophellitol (CP, 6, Fig. 6), another CBE analog, was first isolated from a culture filtrate of the fungus *Phellinus sp.* in 1990 [93] and first chemically synthesized by Tatsuta et al. using a stereospecific

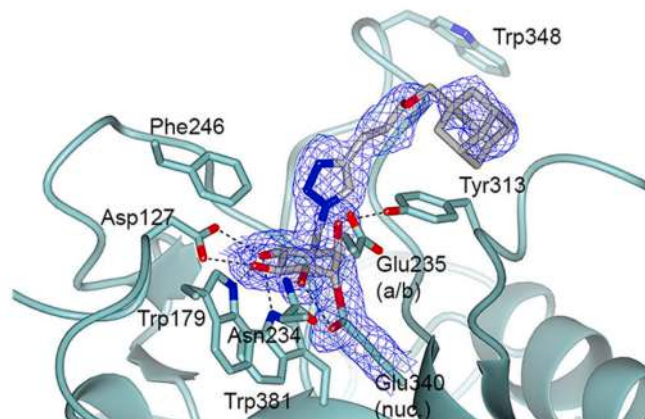


Fig. 7. Structure of GCase with adamantyl-cyclophellitol 15. The linker-adamantyl moiety of 15 is observed in slightly different positions in the two molecules of the asymmetric unit, reflecting its binding through predominantly hydrophobic interactions. Reprinted from Ref. [71]: Artola, M. et al., Functionalized Cyclophellitols Are Selective Glucocerebrosidase Inhibitors and Induce a Bona Fide Neuropathic Gaucher Model in Zebrafish, *J. Am. Chem. Soc.* 2019, 141, 10, 4214–4218, Copyright © 2019 American Chemical Society. This work is licensed under CC BY-NC-ND.

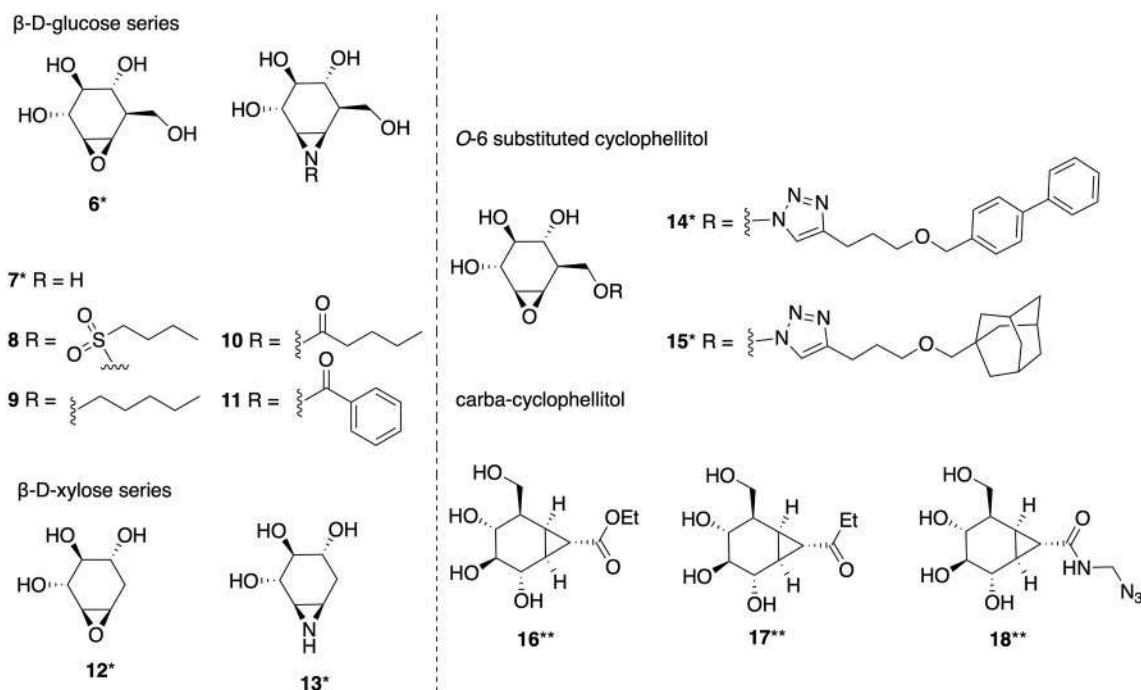


Fig. 6. Chemical structure of cyclophellitol with D-glucose configuration (6) or D-xylose configuration (12), their aziridine analogues with D-glucose (7) or D-xylose configuration (13), and a series of cyclophellitol derivatives. * Irreversible inhibitor, ** Reversible inhibitor (where not specified it has not been determined).

intramolecular cycloaddition reaction between an unsaturated nitrile oxide and an olefin derived from L-glucose [94]. Subsequent approaches have utilized carbohydrates to obtain carbocycles, including initiating a free-radical carbocyclization process from D-glucal [95], employing the Ferrier II reaction from D-mannose [96] or D-glucose [97–99], ring-closing metathesis starting from D-xylose [100,101], and intramolecular cycloaddition from D-glucose [102]. In addition, naturally occurring inositols, such as L-quebrachitol [103] and (–)-quinic acid [104], have also been used for cyclophellitol synthesis, representing ideal sources of chirality and appropriate functional groups.

Early *in vitro* studies on almond β -glucosidase demonstrated that CP with glucose configuration had an IC_{50} of 4.8 μ M, exceeding the inhibitory activity of other compounds such as nojirimycin (**19**, Fig. 8), 1-deoxynojirimycin (Fig. 12), and castanospermine (**22**, Fig. 8), which had IC_{50} values of 14 μ M, 260 μ M, and 63 μ M, respectively [105]. Interestingly, the inhibitory activity of CP was found to be directly proportional to incubation time. Without pre-incubation, 0.57 μ M CP inhibited only 20% of almond β -glucosidase, whereas pre-incubation with CP at room temperature increased inhibition to 70% after 1 h and reached 94% after 3 h [105]. The inhibitory potency of CP is not only time-dependent but also system-dependent. A more recent study demonstrated that CP exhibited different inhibitory concentrations depending on the system used: compared to almond β -glucosidase, for which the IC_{50} value is higher, the IC_{50} values dropped to 30 nM for rhGCase and 86 nM for cultured cells [80]. *In vivo*, using zebrafish larvae, the IC_{50} was 130 nM, suggesting that CP is a potent inhibitor both *in vitro* and *in vivo* (Table 5). Although CP exhibited inhibitory potency in the nanomolar range, a major drawback is that CP-induced inhibition in different systems was similar for both GCase and GBA2, suggesting a lack of selectivity, unlike the promising results obtained with CBE [80].

Changing the CP configuration from glucose to xylose resulted in selectivity advantages both *in vitro* and *in vivo*, as demonstrated by a biological study conducted by Su et al. using rhGCase. This study revealed that, after 3 h of incubation, the IC_{50} of the xylose-configured CP (compound **12**, Fig. 6) was 0.45 μ M, 6-fold lower than that obtained with CBE (2.63 μ M), while in zebrafish larvae the IC_{50} was found to be 3.01 μ M, showing a 50-fold higher selectivity towards GCase over GBA2 (Table 5) [81]. These results suggest that this configurational change represents an important step toward the development of more selective and potent GCase inhibitors.

Considering the inhibitory capacity of CP, several synthetic attempts have been made to explore its derivatives, aiming to find increasingly effective irreversible GCase inhibitors. Similar to CBE, the first structural modification of CP, replacing the epoxide with an aziridine, was performed by Tatsuta et al., in 1991 [106]. The resulting compound (**7**, Fig. 6) with glucose configuration exhibited an IC_{50} of 1.26 μ M against almond β -glucosidase [81,106]. However, its xylose-configured analogue (**16**, Fig. 6) was more potent, with an IC_{50} of 0.24 μ M against rhGCase after 3 h of incubation and an IC_{50} of 6.99 μ M in zebrafish larvae [81], suggesting that aziridine-containing poly-oxygenated cyclohexanes with xylose configuration might be more potent glucosidase inhibitors than their epoxide analogues. These promising results prompted the introduction of structural modification to the aziridine, aiming to mimic the enzyme's natural substrate and

optimize inhibitory efficacy. Starting from compound **7**, the introduction of alkyl (**9**, Fig. 6) or acyl (**10** and **11**, Fig. 6) substituents revealed that the highest inhibitory efficiency was achieved with the allyl substitution [82], suggesting that modifying the basic nitrogen by introducing an amide group leads to reduced inhibitory activity. Similarly, deactivated *N*-sulfonyl analogues showed further reduced inhibitory potency (**8**, Fig. 6) [82]. However, despite the reduction in IC_{50} against rhGCase induced by *N*-substitutions, selectivity for GCase over GBA2 was not observed. To highlight structural features conferring higher GCase specificity compared to other glucosidases, Artola et al. shifted the structural modification from the aziridine to the hydroxyl group at position 6. This choice was based on structural analysis, demonstrating that GCase possesses a cavity formed by hydrophobic residues, such as Tyr313, Phe246, and Trp348, capable of accommodating various hydrophobic substituents linked to O-6 (Fig. 6), a feature absent in other human β -glycosidases (Fig. 7) [71]. The synthesized biphenyl-cyclophellitol and adamantyl-cyclophellitol (**14** and **15**, Fig. 6) showed remarkable potency against rhGCase, with *in vitro* IC_{50} values of 1.06 and 0.96 nM, respectively, approximately 4000-fold greater than CBE and 30-fold greater than unmodified CP. Surprisingly, *in vivo* studies in zebrafish larvae demonstrated IC_{50} values of 4 to 6 nM for **14** and **15** against GCase, indicating not only a potency approximately 7500-fold greater than CBE but also that the value remained within the same order of magnitude from *in vitro* to *in vivo* tests (Table 5). Furthermore, selectivity analysis towards GCase showed that both derivatives had an IC_{50} ratio (GBA2/GCase and recombinant human α -glucosidase (GAA)/GCase) greater than 100,000, making them approximately 4000-fold and 200-fold more selective than CBE [71]. Finally, Beenakker et al. designed a series of carba-cyclophellitols (**16–18**, Fig. 6) [107] that were shown to be GCase inhibitors with minimum concentrations in the micromolar range, but IC_{50} values > 99 μ M [107]. Cyclophellitol derivatives are also extensively utilized in the development of activity-based probes (ABPs) because of their ability to selectively monitor enzymatic activity in biological samples. Unlike traditional methods that quantify total protein levels, ABPs focus on enzymes in their active state, providing crucial insights into biochemical pathways. In a recent study by Radchenko et al. [108], a series of deoxygenated cyclophellitol derivatives were synthesized, with one compound showing selectivity for GCase over GBA2 and GBA3. These complementary ABPs offer potential for evaluating GCase activity in complex biological contexts, enhancing our understanding of its physiological and therapeutic roles.

3.2. Iminoalditols

Although irreversible inhibitors are generally considered more effective for oncology applications, this Review also explores the potential of reversible GCase inhibitors, which may offer greater selectivity compared to other glycosidases, potentially providing advantages. As described above, irreversible inhibitors often exhibit low selectivity, although notable exceptions exist, such as compounds **14** and **15**, which demonstrate nanomolar inhibition potency and high discrimination between GCase and GBA2. Another important consideration is the distinction between *in vitro* and *in vivo* behavior: *in vitro*, using rhGCase,

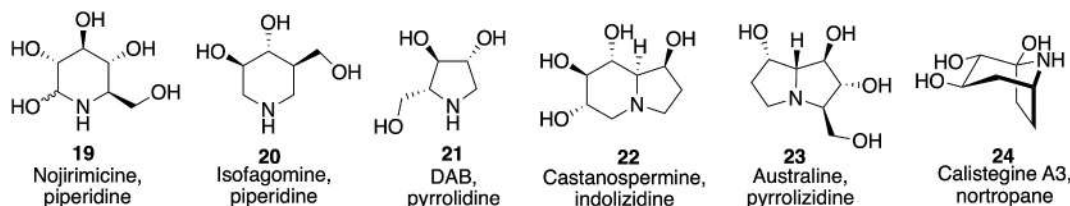


Fig. 8. Chemical structures of representative iminoalditols (iminosugars) (top name) and corresponding main class (bottom name).

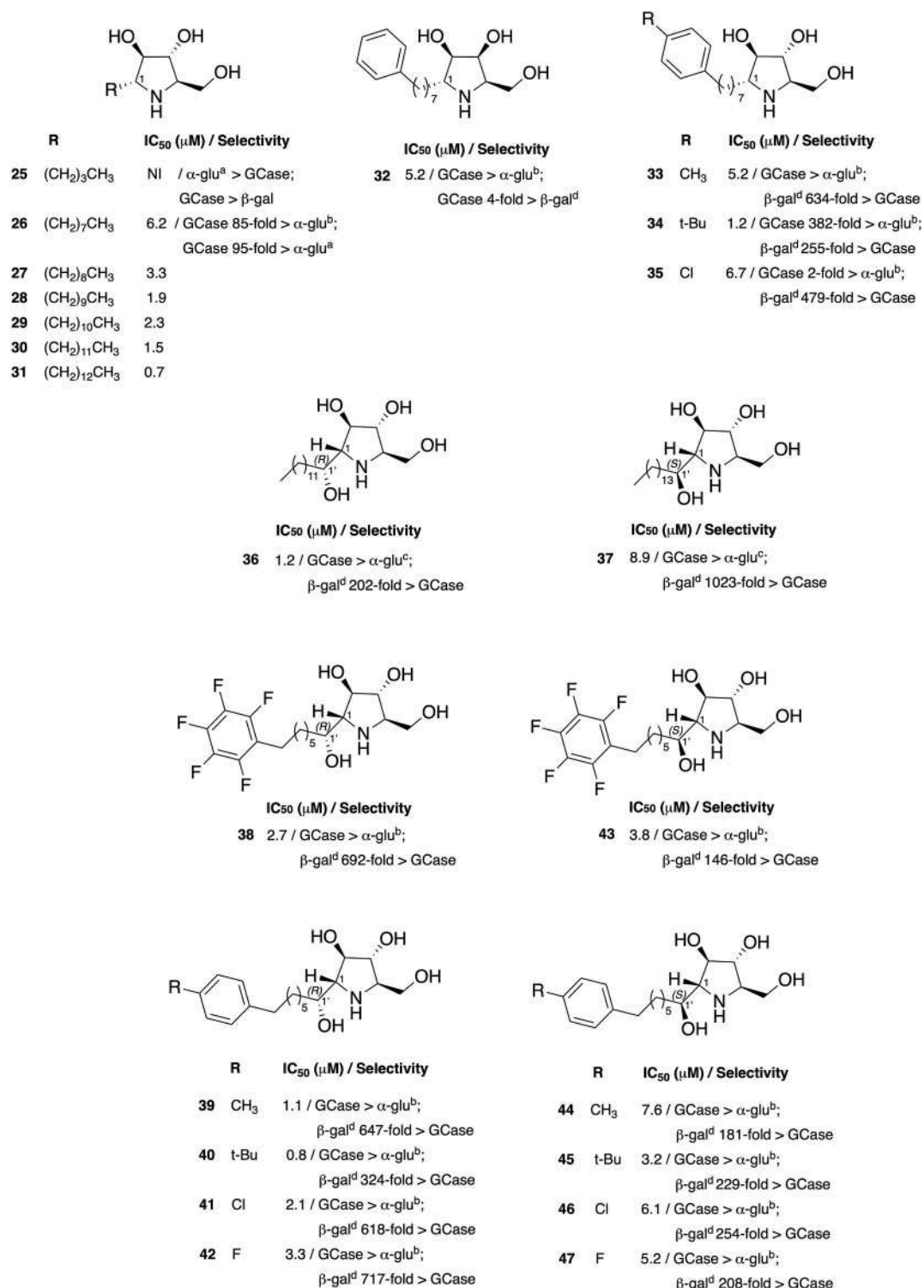


Fig. 9. Chemical structures and inhibitory potency against rhGCase of several DAB and LAB derivatives and other five-membered ring iminosugars, along with their selectivity versus α-glucosidase (α-glu) and β-galactosidase (β-gal). ^asucrase (α-glucosidase) from rat intestinal; ^bmaltase (α-glucosidase) from rat intestinal; ^chuman α-glucosidase; ^dβ-galactosidase from bovine liver.

CBE demonstrates high selectivity for GCase over GBA2, with an 883-fold preference, while the CP is less selective. However, *in vivo*, the selectivity of CBE for GCase decreases significantly, dropping to around 20-fold, highlighting a substantial shift in the inhibitors' ability to discriminate between different targets.

Iminoalditols, also known as iminosugars, are a class of polyhydroxylated alkaloids widely known as GCase inhibitors. They share

structural similarities with conventional carbohydrates but are characterized by the replacement of endocyclic oxygen with a basic nitrogen atom [109,110].

Iminosugars are classified into five structural classes: polyhydroxylated piperidines, pyrrolidines, indolizidines, pyrrolizidines, and nortropans (Fig. 8) [110–112]. In addition to their numerous therapeutic applications [113–115], including antiviral [116–120] and

Table 5Summary of the inhibitory potency (IC₅₀) of CBE, CP, and their derivatives against GCase, both *in vitro* and *in vivo*, along with their respective sources.

Compound	IC ₅₀ (<i>in vitro</i>)	Source of GCase (<i>in vitro</i>)	IC ₅₀ (GCase from Zebrafish larvae)	Selectivity (GCase ^a vs GBA2 or GBA3 ^b)	References
1	4.28 ± 0.50 μM and 2.63 ± 0.34 μM, 0.331 ± 0.189 μM	rhGCase, HEK293T cells	58.5 μM	GCase 883- to 20-fold > GBA2	[80,81]
2	1.63 ± 0.07 μM	rhGCase	-	GCase 6.6-fold > GBA2	[92]
5	0.023 ± 0.003 μM	HeLa	-	-	[92]
6	0.03 ± 0.002 μM	rhGCase	0.130 μM	GCase 6- to 2-fold > GBA2	[80,82]
12	0.45 ± 0.02 μM	rhGCase	3.01 μM	GCase 272-fold > GBA2	[81]
13	0.24 ± 0.03 μM	rhGCase	6.99 μM	GCase 22-fold > GBA2	
7	1.25 and 0.5 μM	Almond	-	β-glucosidase 1.25-fold < GBA2	[106]
8	2.5 μM	rhGCase	-	GCase 8-fold < GBA2 or GBA3	[82]
9	0.017 μM	rhGCase	-	GCase 6-fold < GBA2 GCase 41-fold > GBA3	
10	0.07 μM	rhGCase	-	GCase 3-fold > GBA2 GCase 7-fold > GBA3	
11	1.4 μM	rhGCase	-	GCase 25-fold < GBA2 GCase 14-fold > GBA3	
14	1.06 ± 0.19 nM	rhGCase	5.85 ± 2.44 nM	GCase 10 ⁵ -fold > GBA2 or GCase 1710-fold > GBA2	[71]
15	0.96 ± 0.17 nM	rhGCase	3.84 ± 1.21 nM	GCase 10 ⁵ -fold > GBA2 or GCase 2540-fold > GBA2	

^a Recombinant human GCase.^b Lysates of HEK293T cells expressing GCase, GBA2, and GBA3.

antidiabetic agents [121,122], these glycomimetics have demonstrated promising results in the treatment of lysosomal storage diseases (LSDs). Their interaction with the active sites of specific enzymes allows for stabilizing proper folding of mutant lysosomal enzymes, consequently preventing their cellular clearance [123–125]. The use of iminosugars in cancer research has attracted increasing attention due to their ability to modulate glycosidase activity and interfere with glycosylation processes, which are frequently dysregulated in cancer [126,127]. However, evidence of their antiproliferative activity is currently limited to preclinical studies [128–130], and their therapeutic value has yet to be demonstrated in a clinical setting and the impact on cell proliferation, apoptosis, and the underlying molecular mechanisms are still poorly understood.

3.2.1. Five-membered iminoalditol derivatives

In the field of GCase inhibition, various strategies have been explored to enhance the efficacy of iminoalditols, including optimizing their chemical structure to improve binding affinity. A key factor in this optimization is the modification with long alkyl chains, crucial for obtaining effective GCase inhibitors. These long chains interact favorably with the enzyme's hydrophobic regions, stabilizing the inhibitor-enzyme complex through hydrophobic interactions, thereby improving both efficacy and selectivity [74,83]. For instance, DAB (1,4-dideoxy-1,4-imino-D-arabinitol, Fig. 8) derivatives with alkyl chains longer than octyl at the α-C1 position significantly enhanced inhibitory activity towards GCase as chain length increased. These derivatives not only exhibited stronger inhibition but also demonstrated greater selectivity over α-glucosidase, with IC₅₀ values ranging from 6.2 to 0.77 μM (25 – 31, Fig. 9) [83]. Indeed, α-C1-octyl DAB showed 85- and 95-fold increased selectivity towards GCase compared to maltase and sucrase α-glucosidases from rat intestinal, respectively [83]. Kato and co-workers conducted molecular docking studies to elucidate the structural basis of the competitive inhibition exhibited by these derivatives, focusing on compound 31. Their analysis revealed that the pyrrolidine backbone bound through positive van der Waals interactions with Tyr313 and Trp381, while the hydroxyl groups formed a network of hydrogen bonds with Glu235, Glu340, Asp127, and Trp179. In addition, the tridecyl group fit into a hydrophobic pocket formed by Trp312, Tyr313, Phe316, and Val343, creating favorable interactions that stabilized the orientation of the pyrrolidine ring [83]. In addition to these structural features, replacing the α-C1-octyl side chain of compound 26 with diversely substituted 7-phenylheptyl moieties yielded

compounds 33–35 (Fig. 9), whose potency and selectivity toward rhGCase were markedly improved, as demonstrated by Zi et al. [131]. The contribution of the terminal aryl substituent to the enhanced inhibitory potency is evident when comparing the IC₅₀ values of compounds 33–35 (IC₅₀ = 1.2–6.7 μM) with that of the parent α-C1-heptyl DAB derivative (IC₅₀ = 38 μM) [83]. In particular, the *p*-tert-butyl-substituted aryl derivative 34 combined potent rhGCase inhibition (IC₅₀ = 1.2 μM) with excellent selectivity over α-glucosidase (382-fold). However, although these compounds exhibited increased selectivity for GCase over α-glucosidase from rat intestinal, they lacked specificity towards GCase compared to β-galactosidase from bovine liver. It should be noted that while non-human enzymes, such as bovine liver β-galactosidase and rat intestinal α-glucosidase, are widely used in preclinical medicinal chemistry screenings as surrogate models, these off-target data cannot be considered fully representative of the selectivity profile against human glycosidases relevant to cancer therapy.

Conversely, Gao et al. recently evaluated the selectivity of a library of DAB derivatives toward human GCase over human α-glucosidase [132]. In particular, the introduction of a hydroxyl group at the C-1' position of the lipophilic C-1 pendant was reported, systematically varying both the alkyl chain length (from C5 to C15) and the absolute configuration at the C-1' stereocenter. The tridecyl derivative bearing the *R* configuration at C-1', compound 36 (Fig. 9), exhibited an IC₅₀ value against rhGCase comparable to that of its non-hydroxylated analogue 31 (IC₅₀ = 1.2 μM vs 0.7 μM), while demonstrating an outstanding selectivity over human α-glucosidase (>833-fold). Within the epimeric series bearing the *S* configuration at C-1', several compounds showed no detectable inhibition of human α-glucosidase (0% inhibition at 1 mM). Among them, the 1'-hydroxypentadecyl derivative 37 displayed the highest potency against human GCase, with an IC₅₀ value of 8.9 μM (Fig. 9).

A similar trend was also observed for the two C-1' epimeric series in analogous compounds bearing, in addition to the C-1' hydroxyl group, a terminal aryl moiety on the heptyl substituent attached to the C-1 position of the DAB skeleton (compounds 38 to 47, Fig. 9). Enhanced inhibitory potency is attributed to the *R*-configuration of the C-1' stereocenter, which influences affinity for the GCase binding site, with the *R*-configuration binding more strongly than the *S*-configuration [133], thus optimizing overall inhibitory activity (38 vs 43, 39 vs 44, 40 vs 45, 41 vs 46, 42 vs 47, Fig. 9) [131]. Molecular docking studies, using the X-ray structure of human acid GCase, revealed that the pyrrolidine ring binds similarly in the active site, but the hydrophobic chains adopt different orientations [131]. Compounds 38 and 40 occupied the same

hydrophobic groove, while **33-35**, **39**, **41**, and **42** occupied a different one. These different hydrophobic groove occupancies did not significantly influence their inhibition profiles. While the addition of a hydroxyl group at C-1' did not directly influence the interactions within the binding sites, it altered pyrrolidine ring conformations and chain orientations, enhancing GCase inhibition in the *R*-configuration [131]. Compounds **45** and **47**, with *p*-fluoro and *p*-tert-butyl groups, also exhibited different chain orientations compared to **43**, **44**, and **46**, but similar DAB conformations and hydrogen bonding patterns. The lower GCase inhibition observed for compounds **43-47** compared to their C-1' epimers could be due to less efficient accommodation within the hydrophobic grooves [131]. Unfortunately, despite the increased affinity toward GCase, these compounds remain more selective toward β -galactosidase than GCase, highlighting the need for further structural modifications to improve specificity.

The incorporation of a terminal aromatic tetralin group into the C-1 alkyl chain, as in 7-(tetralin-2-yl)-heptyl-DAB (**48**, Fig. 10), resulted in an approximately 8-fold decrease in IC_{50} compared to compound **26** (from 6.2 μ M to 0.82 μ M) [72]. This is likely attributed to the size and morphology of the hydrophobic GCase binding site, which includes the vestibular pocket and hydrophobic groove, exerting precise control over ligand binding affinity, as evidenced by molecular docking and molecular dynamics simulations. The presence of aromatic amino acid residues within the hydrophobic groove notably enhances the affinity for ligands bearing a terminal aromatic tetralin group. This suggests that further increasing compound activity may be achievable by introducing aromatic interactions alongside existing hydrophobic interactions [72].

Following modifications at the C-1 position, substitutions at the C-4 position of DAB were performed to analyze the structure-activity relationship and determine whether the new synthesized derivatives exhibited improved GCase inhibition. For instance, removing the CH_2OH group at the C-4 position in the DAB derivative (**49**, Fig. 10) increased its inhibitory potency 8-fold against GCase through a non-competitive mechanism (K_i 0.40 μ M) compared to **26** [134]. Conversely, replacing the C-4 hydroxymethyl group with aryl chains on unmodified DAB again demonstrated the need for a hydrophobic moiety to impart selectivity towards GCase over α -glucosidase. Furthermore, the presence of a lipophilic group at the *para* position enhanced inhibitory potency against GCase (**52-54**, Fig. 10) [135]. All compounds formed hydrogen bonds with residues Trp179, Asp127, and Trp381, along with π - π stacking interactions with residue Trp313. Interestingly, when a fluorine atom was introduced at the *para*-position of the phenyl group (**52**, Fig. 10), it fit into the hydrophobic pocket formed by residues Phe347 and Phe397, resulting in not only weak hydrogen bonding interactions with the aromatic hydrogens of these residues, but also in an atypical anion- π interaction with the Phe397 residue. Further improvement of the binding affinity towards the hydrophobic pocket was achieved by substituting the fluorine at the *para*-position with methyl and phenyl groups (**53** and **54**). In contrast, LAB (1,4-dideoxy-1,4-imino-L-arabinitol, the DAB enantiomer) derivatives **50** and **51** failed to form similarly strong and consistent interactions, confirming the key role of stereochemistry [135].

Summarizing the results observed so far leads to the conclusion that structural modification on DAB has a clear advantage over those on LAB

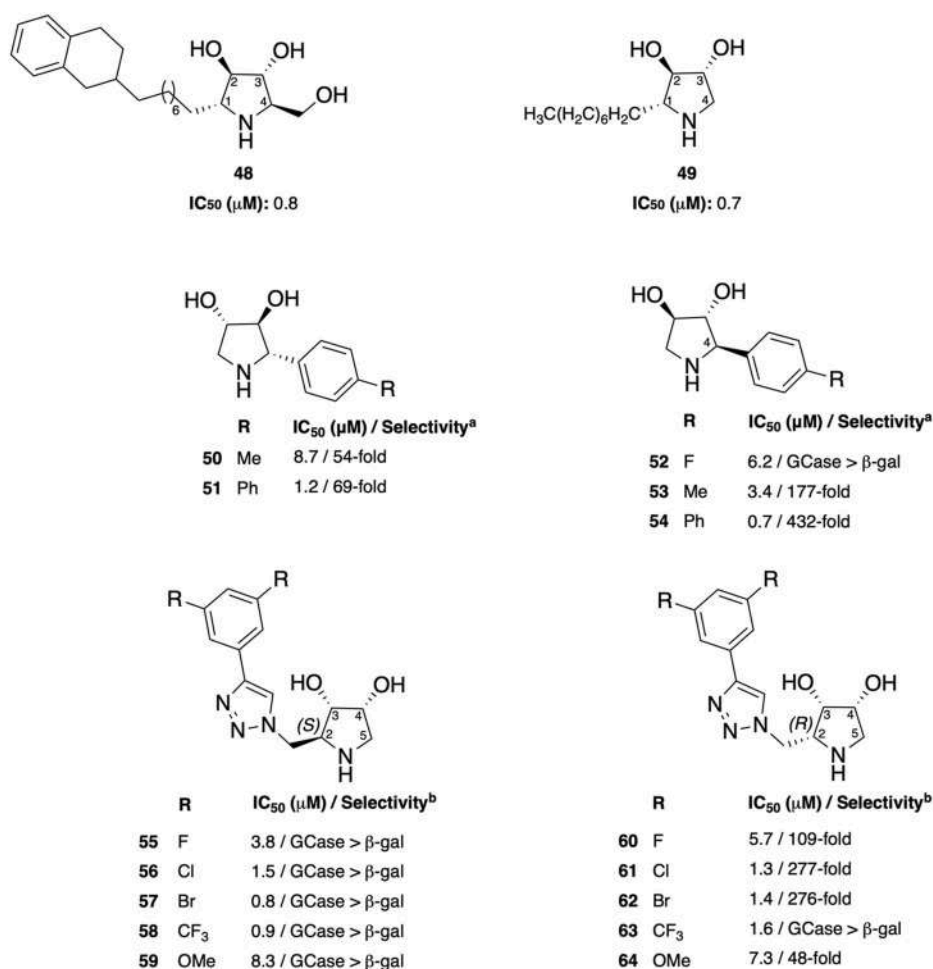


Fig. 10. Chemical structures and inhibitory potency against rhGCase of several DAB and LAB derivatives and other five-membered ring iminosugars, along with their selectivity versus β -galactosidase (β -gal). ^aGCase vs β -galactosidase from bovine liver; ^bGCase vs human β -galactosidase A.

in achieving greater GCCase inhibition potency. The presence of a long alkyl chain at C-1 on the glycomimetic is essential for achieving strong GCCase inhibition, while the addition of an aromatic interaction at C-4, leveraging the affinity of aromatic amino acid residues within the hydrophobic groove, can further enhance the inhibitory potency of the final compound [135]. These combined structural features offer a promising approach for optimizing the effectiveness of GCCase inhibitors.

Martínez-Bailén et al. [73] synthesized a library of 3,4-dihydroxy-pyrrolidine derivatives by reacting two epimeric dihydroxylated azido-methylpyrrolidines with differently substituted phenylacetylenes to study the effect of the substitution of the phenyl moiety on GCCase inhibition efficacy. Overall, 3,5-disubstituted derivatives exhibited higher inhibitory potency compared to *para*-substituted and unsubstituted compounds, with derivatives 55–59 (Fig. 10) being the most bioactive, showing IC₅₀ values ranging from 1.5 to 0.83 μ M (Fig. 10). The presence of halogens in the 3,5-disubstitution pattern significantly enhanced inhibition potency, up to 10-fold in the most relevant examples (55–59 and 60–64, Fig. 10) [73]. Nevertheless, GCCase showed no discrimination between the two C-2 pyrrolidine epimers, as their activities were very similar, suggesting that the aromatic moiety primarily influenced activity. However, differences between the C-2 epimers were observed in selectivity towards GCCase compared to human α -galactosidase A, with compounds 60–64 (Fig. 10) being weak inhibitors of the latter enzyme [73]. Molecular docking confirmed key interactions already described: the pyrrolidine nitrogen forms hydrogen bonds with Glu235 and Glu340, the C-4 hydroxyl aligns with a hydrophilic pocket, and the C-3 hydroxyl binds with Asp127 and Asn396 (Fig. 11). The inhibitor's remaining structure extended into a hydrophobic groove. In particular,

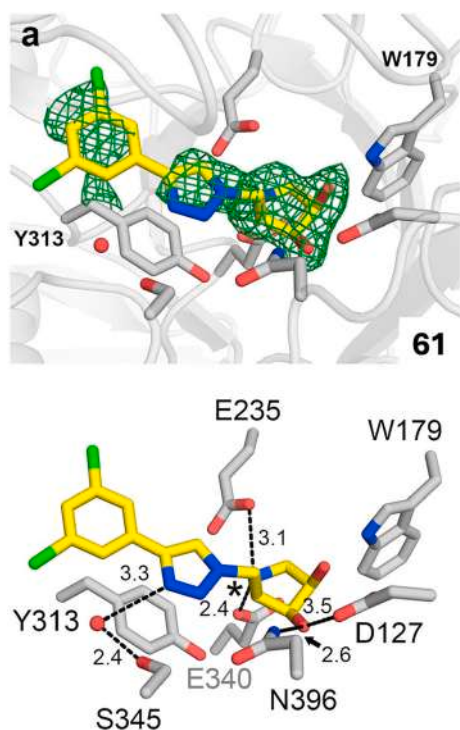


Fig. 11. Binding interactions of pyrrolidine-triazole **61** in GCCase active site. Above: green mesh represents Fourier difference maps Fo-Fc, calculated from the refined protein model prior to addition of ligands and contoured at 3 σ . The active sites are oriented relative to Tyr313 and Trp179. Below: individual interacting residues are labeled including hydrogen bond interactions (dashed lines, Å). The key chiral carbon center which distinguishes **61** (2*R*) is marked with an asterisk. Reprinted from Ref. [73]: Martínez-Bailén, M. et al., Exploring Substituent Diversity on Pyrrolidine-Aryltriazone Iminosugars: Structural Basis of β -Glucocerebrosidase Inhibition, *Bioorg. Chem.* 2019, 86, 652–664, Copyright © Elsevier.

the triazole moiety contributed significantly to binding, establishing a water-mediated interaction with Ser345, while the side chain of Tyr313 participated in π - π stacking. Although the phenyl group exhibited a weak interaction with GCCase, the structure-activity relationship study highlighted its importance in binding, suggesting a contribution to binding energy despite its limited involvement in direct interactions [73].

3.2.2. Six-membered iminoalditol derivatives

Piperidine iminosugars are probably the most studied class of GCCase inhibitors, owing to their promising results in the treatment of Gaucher disease, where they have demonstrated effectiveness in stabilizing mutated GCCase enzyme forms. A recent Review highlights how glycosidase inhibition *in vitro* is significantly influenced by substituents at the C-1 and C-5a positions of polyhydroxylated piperidine isoiminosugars, compared to their unsubstituted analogues and common iminosugars [136], also examining the impact of nitrogen relocation, from the position of the endocyclic oxygen in monosaccharides to the anomeric carbon, leading to the formation of isoiminosugars. Here, we focus on selected examples that exhibit strong GCCase inhibition in the nanomolar range, including novel scaffolds that diverge from the previously characterized archetypes.

First-generation compounds, such as 1-deoxynojirimycin (DNJ, Fig. 12), exhibited limited inhibitory activity, with an IC₅₀ of 240 μ M for GCCase [137]. This limited inhibition highlighted the need to improve the binding affinity and potency of these inhibitors, leading to the development of second-generation compounds characterized by targeted structural modifications, such as the introduction of hydrophobic *N*-alkyl and α -C1-alkyl chains. These additions significantly increased inhibitory activity, reducing IC₅₀ values to the low micromolar and, in some cases, even nanomolar range [138].

Similarly, 1,5-dideoxy-1,5-imino-D-xylitol (DIX, Fig. 12) gains inhibitory potency in the nanomolar range upon modification at the α -1-C position with variable-length alkyl chains, despite initially presenting an IC₅₀ value of 2.3 μ M against GCCase [137]. In a study by Wolfsgruber et al. [84], a series of *N*-alkylated iminosugars based on DIX and DNJ backbones were examined (Fig. 12). The results showed that all tested compounds inhibited GCCase, but those with the D-glucose configuration (65a–f, Fig. 12) demonstrated higher activity against rhGCCase compared to their D-xylose analogues (66a–f, Fig. 12). Notably, none of the compounds significantly inhibited human lysosomal α -galactosidase, but all compounds inhibited bovine liver β -galactosidase in the micromolar range, indicating a lack of selectivity towards GCCase [84]. Sevsšek and co-workers [70] introduced a series of *N*-substituted DNJ derivatives, synthesizing a range of bicyclic isourea derivatives (67–71, Fig. 12). This new group of GCCase inhibitors exhibited significantly high inhibitory potency against rhGCCase in the nanomolar range at pH 5.2 (IC₅₀ = 135.5–14 nM, Fig. 12). Potency increased with alkyl chain elongation, consistent with previous studies, suggesting that the extended alkyl chain's favorable interactions with enzyme's hydrophobic pocket amplified efficacy [70]. Moreover, these inhibitors exhibited high selectivity for GCCase over β -galactocerebrosidase (GALC, IC₅₀ > 10 μ M). Sevsšek et al. continued their investigation by introducing potent GCCase inhibitors derived from DIX (72–76, Fig. 12). Notably, guanidinium analogues exhibited strong selectivity as human GCCase inhibitors, showing low nanomolar IC₅₀ values ranging from 17 to 245 nM (Fig. 12). The selectivity of these guanidinium derivatives was confirmed by their inability to inhibit human recombinant GALC, for which IC₅₀ values exceeded 10 μ M, thus demonstrating a high degree of selectivity for GCCase. Molecular docking studies revealed that the guanidinium moiety established additional cation- π interactions with Tyr313 compared to the smaller amine function of *N*-nonyldeoxynojirimycin (NN-DNJ). Additionally, the guanidinium group formed a hydrogen bond/salt bridge with Glu235, a stabilizing interaction that is absent in the NN-DNJ complex, revealing higher binding affinity, consistent with the inhibition data [139]. Recently, a series of

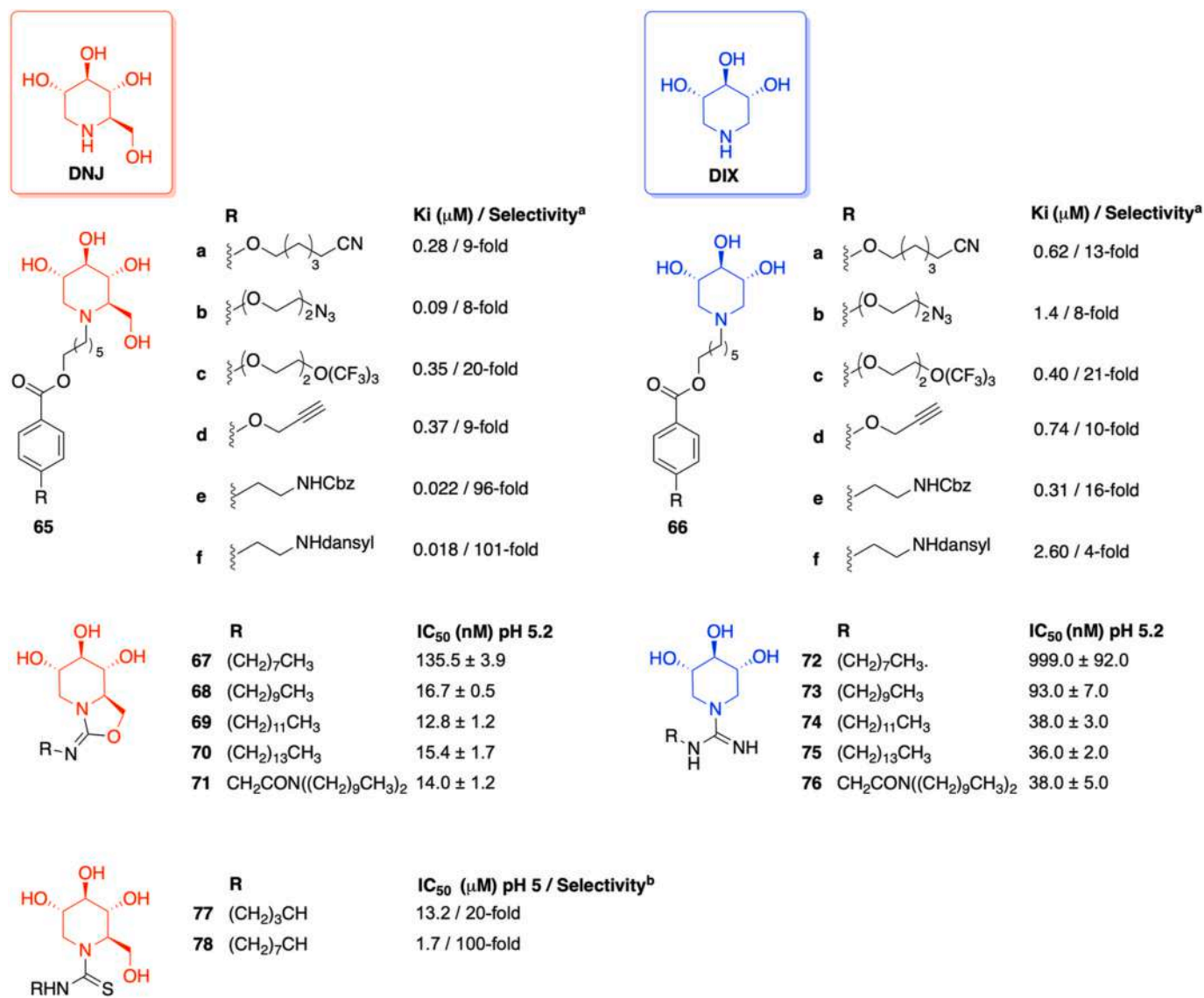


Fig. 12. Chemical structure, inhibitory potency against rhGCase of DNJ- and DIX-aminosugar derivatives, along with their selectivity versus α -glucosidase (α -glu) and β -galactosidase (β -gal). ^aGCase vs human α -glucosidase; ^bGCase vs β -galactosidase from bovine liver.

DNJ-based *sp*²-aminosugar thioureas bearing various aglycone substituents was developed to replace the basic amine with a neutral thiourea moiety, aiming to selectively modulate GCase activity over α -glucosidase (α -glu) [140]. The design preserves a tunable exocyclic H-bond (NH), whose acidity and interaction with the aglycone pocket are governed by the *N*-substituent, thereby linking structural variation to enzymatic selectivity. In this context, aliphatic electron-donating groups weaken H-bond donation while simultaneously increasing hydrophobicity, which favors GCase binding. Conversely, electron-withdrawing aromatic substituents enhance the acidity of the H-bond donor and enable π -stacking interactions, thereby promoting α -glu binding. Consistent with this rationale, the *N*-butyl (77, Fig. 12) and *N*-octyl (78, Fig. 12) derivatives inhibited GCase in the low-micromolar range (IC_{50} = 13.2 μ M and 1.7 μ M, respectively), with selectivity increasing (approximately 20- to 100-fold over α -glu) with the lengthening of the alkyl chain, a trend previously observed in related scaffolds [140].

As already highlighted for pyrrolidines, the introduction of aromatic groups significantly increases inhibitory activity towards GCase in six-membered ring aminosugars as well, likely due to the formation of π - π interactions with aromatic residues in the hydrophobic pocket of the

enzyme. Liu et al. synthesized a series of novel benzimidazole-aminosugars, characterized by a substituted phenyl group on the benzimidazole's benzene ring (79a-p and 80a-p, Fig. 13) [141]. Tested against almond β -glucosidase, these compounds demonstrated potent inhibitory properties with IC_{50} values ranging from 30 to 4680 nM (Fig. 13). Molecular docking revealed that the piperidine ring fits into the active site of the enzyme similarly to unmodified DNJ. A hydrogen bond and a salt bridge formed by the imidazole NH with Glu405 were observed, as well as a π - π stacking between the imidazole ring and residues Trp406 and Trp122. Although a portion of the benzimidazole's benzene ring was exposed to the solvent, it could still form a π - π stack with Trp324, making these benzimidazole aminosugars particularly well-suited for binding to the active site pocket [141].

In optimizing affinity and selectivity towards GCase, structural modifications on the nitrogen atom are not the only effective approach. Introducing alkyl chains on carbons adjacent to the nitrogen or on hydroxyl groups also contributes substantially to optimization. Baudoin-Dehoux et al. investigated the inhibitor activity of C- and O-alkyl aminosugars towards GCase, focusing on pyrrolidine and piperidine structures [79]. Their results demonstrated that replacing one hydroxyl group with a C-alkyl chain in a DIX derivative (84 and 85, Fig. 14),

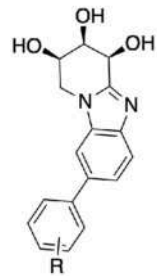
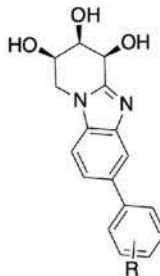
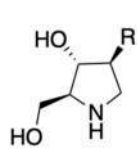
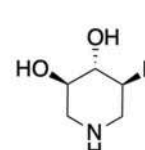
	R	IC ₅₀ (nM)		R	IC ₅₀ (nM)
	a H	1580 ± 60		a H	N.d.
79a-p	b 2-CH ₃	270 ± 30	80a-p	b 2-CH ₃	8730 ± 120
	c 4-OCH ₃	910 ± 20		c 4-OCH ₃	N.d.
	d 3,4-diOCH ₃	80 ± 10		d 3,4-diOCH ₃	60 ± 10
	e 3,4,5-triOCH ₃	3200 ± 530		e 3,4,5-triOCH ₃	30 ± 10
	f 3-F; 4-OCH ₃	300 ± 40		f 3-F; 4-OCH ₃	590 ± 130
	g 3-Cl; 4-OCH ₃	1240 ± 50		g 3-Cl; 4-OCH ₃	330 ± 60
	h 3-CH ₃ ; 4-OCH ₃	760 ± 90		h 3-CH ₃ ; 4-OCH ₃	1760 ± 310
	i 3,5-diCH ₃ ; 4-OCH ₃	180 ± 0		i 3,5-diCH ₃ ; 4-OCH ₃	70 ± 10
	j 4-OH	690 ± 50		j 4-OH	560 ± 10
	k 3,4-diOH	780 ± 50		k 3,4-diOH	250 ± 10
	l 3,4,5-triOH	1930 ± 40		l 3,4,5-triOH	4680 ± 770
	m 3-F; 4-OH	1430 ± 70		m 3-F; 4-OH	390 ± 20
	n 3-Cl; 4-OH	420 ± 60		n 3-Cl; 4-OH	30 ± 0
	o 3-CH ₃ ; 4-OH	330 ± 20		o 3-CH ₃ ; 4-OH	120 ± 10
	p 3,5-diCH ₃ ; 4-OH	510 ± 110		p 3,5-diCH ₃ ; 4-OH	60 ± 10

Fig. 13. Chemical structure and inhibitory potency vs almond β -glucosidase of benzimidazole-iminosugars.

	R	IC ₅₀ (nM)		R	IC ₅₀ (nM)
	81 <i>n</i> -Bu	>8000		84 <i>n</i> -Bu	108
	82 <i>n</i> -Oct	4200		85 <i>n</i> -Oct	11
	83 <i>On</i> -Oct	6000		86 <i>On</i> -Hept	4

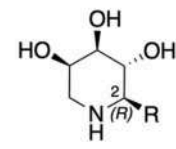
	R	IC ₅₀ (μ M)
	87 (CH ₂) ₈ CH ₃	8.0 ± 1.0
	88 (CH ₂) ₁₀ CH ₃	7.0 ± 1.0
	89 (CH ₂) ₁₁ CH ₃	1.5 ± 0.1
	90 (CH ₂) ₁₂ CH ₃	9.0 ± 2.0

Fig. 14. Chemical structure of iminosugars C- and O-alkyl pyrrolidine and piperidine.

rather than in a pyrrolidine (**81** and **82**, Fig. 14), significantly increased inhibitory potency, reaching the nanomolar range. This suggests that two hydroxyl groups are sufficient for effective GCase inhibition, providing a simplified structural template for potent inhibitors [79]. A similar effect was observed with the introduction of an O-alkyl chain. Notably, this modification in five-membered ring iminosugars (**83**, Fig. 14) did not decrease IC₅₀, whereas in six-membered ring iminosugars (**86**, Fig. 14), it resulted in a 3-fold improvement in inhibitory potency [79].

Davighi et al. made similar observations [142]. They found that, regardless of the absolute configuration of the hydroxyl group in the trihydroxypiperidine backbone, it is essential to insert an alkyl chain of at least eight atoms on the hydroxyl group at position 3 or to replace the hydroxyl group with an additional amine containing a conjugated alkyl chain to maintain effective GCase inhibition [142].

Clemente et al. synthesized C-2 substituted (3*R*,5*R*)-3,4,5-trihydroxypiperidines with various alkyl chains (**87–90**, Fig. 14). They found that relocating the alkyl chain from the endocyclic nitrogen to the C-2 position significantly enhanced inhibitory efficiency [69] compared to the corresponding N-alkyl derivatives [133,143]. Specifically, when the configuration at C-2 is *R*, the IC₅₀ values towards GCase extracts from human leukocyte were more than 100 times lower than the analogues with the *S*-configuration at C-2 [74]. Additionally, a preliminary biological screening of the compounds at 1 mM inhibitor concentration was conducted to assess selectivity against a panel of human lysosomal enzymes extracted from human leukocytes isolated from healthy donors, i.

e. GCase, α -L-fucosidase, α - and β -galactosidase, α - and β -mannosidase, and α -glucosidase. The results clearly indicate that compounds **87–90** are selective GCase inhibitors, inhibiting GCase activity at 1 mM. Although some inhibition of α -L-fucosidase was observed at this concentration, the compounds displayed markedly lower activity toward the other lysosomal glycosidases tested [74]. These findings align with previous research demonstrating that alkylated inhibitors, particularly those with longer chains, exhibit superior inhibition profiles due to their enhanced ability to interact with hydrophobic regions of the enzyme's active site [83,137]. Clemente et al. observed a similar trend in GCase inhibition using a trihydroxypiperidine with two aliphatic chains substituted at nitrogen and carbon positions [144]. Molecular docking studies indicated that the piperidine ring adopts an orientation in the active site comparable to isofagomine, while stereogenic center alterations within the alkyl chain led to changes in the hydrophobic groove occupancy [144].

Isofagomine (IFG, **20**, Fig. 8) and its analogues exhibit promising inhibitory activities against GCase. Recently, Li et al. [145] designed a collection of C5a *S*- and *R*- aminomethyl IFG-based derivatives (**91a–e**, **92a–d**, **92f**, Fig. 15) that demonstrated rhGCase inhibition with IC₅₀ values in the nanomolar range. In this case, the IC₅₀ value was mainly influenced by the nature of the substituent introduced at the C5a position rather than by its absolute configuration, as previously observed.

In addition, prodrug strategies based on pH-sensitive groups, such as acetals or orthoesters, have been reported to enhance bioavailability at the target site while minimizing systemic toxicity [146–149]. In parallel,

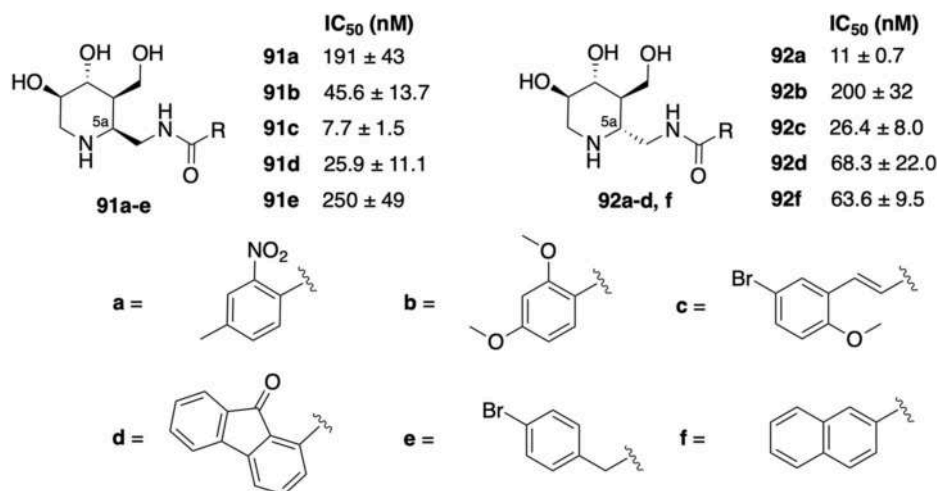


Fig. 15. Chemical structure and inhibitory potency vs rhGCase of several IFG derivatives.

the incorporation of antioxidant functionalities has enabled the development of dual-action iminosugars that combine ROS-scavenging capabilities with lysosomal enzyme modulation [147,150]. This integrated approach is particularly attractive as it simultaneously addresses oxidative stress and enzymatic dysregulation, two interconnected processes involved in disease progression. Accordingly, this dual pharmacological profile holds significant potential for therapeutic applications in oncology.

In this context, selenourea-containing iminosugars (**93a-c** and **94a-c**, Fig. 16) showed inhibition of GCase in the low-micromolar range, with the selenourea moiety acting as crucial pharmacophore for antioxidant activity [151]. Interestingly, the radical scavenging properties were not significantly affected by variations in alkyl chain length or phenyl substitution, evidencing robust redox behavior across the entire series.

3.2.3. Multivalent iminosugars

Glycosidase inhibition has been improved by designing multivalent frameworks that connect multiple bioactive units to a molecular scaffold [152,153], such as fullerenes [154,155], aromatics [156,157], nanoparticles [158–161], dendrimers [162], and polymers [163–165]. The multivalent effect, first observed in carbohydrate-lectin interactions, describes the increased biological response of compounds with multiple bioactive units compared to monovalent compounds towards the protein receptor [153,166,167]. A positive multivalent effect occurs when the relative potency (rp) of the ligands is higher in a multivalent system compared to the corresponding monovalent one [153,168]. The relative potency of a multivalent inhibitor is determined by dividing the IC₅₀ of

the monovalent reference compound by the IC₅₀ of the multivalent system (IC₅₀ monovalent reference/IC₅₀ multivalent system). Usually the rp/n (n = number of binding units) is also calculated to quantify the multivalent effect per bioactive unit, considering the valency of the multivalent ligand. This effect can result from several phenomena that affect overall binding affinity, beyond the enhanced local concentration around the binding site (the “statistical rebinding effect”). These phenomena include interaction with multiple identical binding sites on the target (the “chelation effect”), influence of binding selectivity at different binding sites (the “subsite binding effect”), and favorable association of multiple targets (the “clustering effect”) [153]. Although glycosidases display single and poorly accessible substrate-binding sites, it is now recognized that these enzymes can also be subjected to the multivalent effect [169]. Specifically, GCase is suggested to exist in a concentration-dependent monomer-dimer equilibrium [170], and specific modulators can promote GCase dimerization [171]. This observation, along with the presence of an allosteric binding site, suggests that the chelation effect, involving multivalent ligand binding to oligomeric receptors, may also be operative. Multivalent iminoalditols have demonstrated effectiveness in reducing IC₅₀ values against GCase. For example, multivalent DNJ-based systems (**95** and **96**, Fig. 17) showed 480-fold and 2200-fold improvements in IC₅₀ values towards GCase compared to monovalent DNJ [172].

More recently, Borie-Guichot et al. synthesized a series of multivalent pyrrolidine-based compounds utilizing cyclophosphazene-based dendrimers and a pentaerythritol scaffold (**99–102** and **105–108**, Fig. 18) [173]. Compared to the monovalent reference compounds **97**

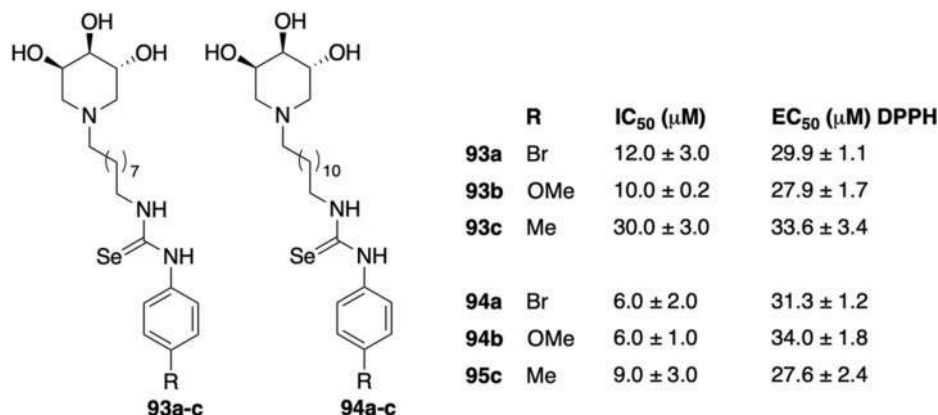


Fig. 16. Chemical structure of selenourea-iminosugar derivatives.

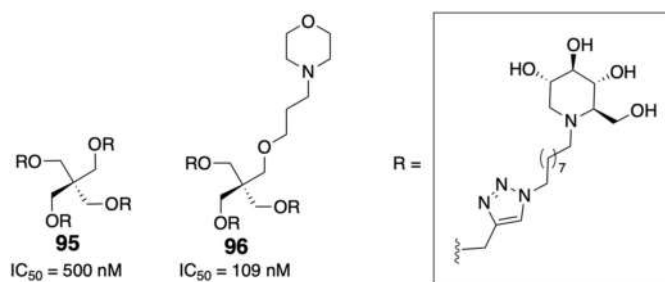


Fig. 17. Chemical structure of DNJ-derived multivalent compounds.

and **98** ($IC_{50} = 0.463 \pm 0.022 \mu M$ and $1.799 \pm 0.296 \mu M$, respectively), the cyclotriphosphazene-based multivalent iminosugars demonstrated significantly enhanced GCCase inhibition as valency increased, with 26-fold or 9-fold (**99** and **100**) and 96-fold or 35-fold (**101** and **102**) improvements. Similarly, pyrrolidine-based multivalents, using pentaerythritol as a scaffold, achieved 10-fold or 20-fold (**105** and **106**) and 26-fold or 48-fold (**107** and **108**) improvements relative to their corresponding monovalent compounds **103** and **104** ($IC_{50} = 6.6 \pm 0.4 \mu M$ and $105.1 \pm 14.0 \mu M$, respectively). The study demonstrated that 12-valent cyclotriphosphazene-based dendrimers and a 6-valent pentaerythritol scaffold are the most effective in enhancing GCCase inhibition, providing significantly greater inhibitory potency than lower-valent systems [173]. Additionally, this study showed that the monofluorocyclooctatriazole group favorably enhanced ligand affinity for GCCase. More generally, iminosugar derivatives containing a monofluorocyclooctatriazole group exhibited greater GCCase inhibitory potency compared to their corresponding triazole analogues. Molecular dynamics studies revealed that the regioisomers, despite being larger, formed additional stabilizing interactions compared to compound **103**, which contained only a triazole group [173]. As previously described for monovalent compounds, GCCase also discriminates between epitopes presented on a multivalent platform. Notably, the *R* configuration at the C-5 position of the pyrrolidine ring consistently induces stronger enzyme affinities (Fig. 18). This observation emphasizes the importance of stereochemistry in multivalent inhibitor design, as even minor configuration alterations can significantly impact binding efficiency and overall inhibitory potency. Martínez-Bailén et al. corroborated this observation by synthesizing a series of pyrrolidine-based multivalent iminosugars [174]. They studied the impact of iminosugar type, scaffold valency, and the connection between the inhiotope and central scaffold. Derivatives characterized by a pyrrolidine-aryltriazole hybrid iminosugar showed selective and potent GCCase inhibition, with increasing potency from trivalent to nonavalent clusters (**109–116**, Fig. 18). The multivalent **2S** derivatives showed 232-fold and 59-fold greater GCCase selectivity compared to α -galactosidase (**112**, **114**, Fig. 18). In contrast, the corresponding multivalent **2R** derivatives reduced GCCase selectivity to 5- to 3-fold compared to α -galactosidase (**109**, **111**, **113**, **115**, Fig. 18) [174].

Vanni et al. [175] also developed multivalent systems based on six-membered iminosugars using propargylated scaffolds and a CuAAC reaction with an azido-terminated trihydroxypiperidine (**117–121**, Fig. 19). Their study demonstrated that these multivalent inhibitors were significantly more potent than their monovalent counterpart ($IC_{50} = 500 \pm 50 \mu M$). Notably, while compound **120** was the most effective inhibitor, with an IC_{50} of $6 \pm 2 \mu M$ against GCCase from human leukocyte extracts, compound **118** exhibited the highest rp/n ratio, indicating its superior efficiency per bioactive unit. Furthermore, the topological orientation of the bioactive units within the multivalent architecture played a crucial role in determining inhibitory performance, as evidenced by the differences observed between trivalent (**117** and **118**, Fig. 19) and hexavalent (**120** and **121**, Fig. 19) systems with varying central scaffolds.

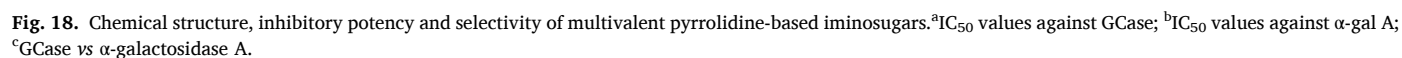
Utilizing the same bioactive unit but adopting a different synthetic strategy, a series of divalent and trivalent glycoclusters were prepared via a reductive amination approach (RDA), all comprising a central aromatic scaffold [176]. These multivalent derivatives (**123**, **124**, and **125**, Fig. 19) exhibited strong inhibitory activity toward GCCase, with IC_{50} values in the 8–20 μM range. A critical observation concerned the role of the central aromatic moiety: its mono-substitution is insufficient to confer activity, as highlighted by the lack of inhibition for the analogue **122**, indicating that enzymatic affinity is governed not only by the chemical nature of the functional groups but also by their spatial arrangement and their ability to engage in multivalent interactions.

In this context, the superior performance of compounds **123**, **124**, and **125** arises from their multivalent architecture, which facilitates cooperative binding and enhances complex stability, a benefit that remains even after normalization for valency. Kinetic analyses further revealed that both the stoichiometry and the spatial distribution of the iminosugar units critically influence the inhibition mode. While the divalent compound **123** acts as a competitive inhibitor, the analogues **124** and **125** display a mixed-type mechanism, suggesting the involvement of secondary binding regions beyond the catalytic site. Interestingly, the closely related analogue **118** remains a purely competitive inhibitor, despite differing only by a slightly longer linker. Computational docking indicates that this subtle structural variation prevents **118** from accessing a channel that connects the catalytic pocket to a secondary cavity. This channel remains accessible to compound **125**, allowing for simultaneous interaction with both the catalytic and allosteric sites.

Stauffert et al. synthesized a series of DIX-derived heterodimers and homodimers based on a pegylated scaffold, along with monovalent compounds [177]. These new compounds were shown to be potent GCCase inhibitors, exhibiting nanomolar potency (**126–135**, Fig. 20). Interestingly, except for compounds **126** and **129**, which exhibit micromolar inhibitory potency, the other compounds demonstrated significant nanomolar inhibitory activity against rhGCCase. Notably, the mechanism of inhibition varies: most compounds inhibit the enzyme non-competitively, while compounds **132** and **134** operate through a competitive mechanism [177]. The highest inhibitory activity was observed when the enzymatic inhibition assay was conducted at neutral pH, revealing higher K_i values at pH 5.2 compared to pH 7.0 (Fig. 20), indicating a higher affinity for GCCase of the endoplasmic reticulum [177]. This is crucial, as the use of these molecules as pharmacological chaperones could be advantageous, given the need for correct folding of misfolded GCCase to perform its enzymatic function in the lysosome. However, when GCCase inhibition is required due to enzyme over-expression in various cancers, the compounds' lack of selectivity towards different β -glucosidase isoforms may be problematic. Non-selective inhibition may compromise other essential enzymatic functions unrelated to the therapeutic target, potentially leading to unwanted side effects and reducing overall treatment efficacy.

Furthermore, by varying the type of scaffold used in multivalent systems, researchers can achieve distinct properties, such as the development of light-activated inhibitors leveraging a photo-controlled strategy to enhance therapeutic precision [178–181]. This approach involves attaching photosensitive moieties, such as azobenzene (AZB) or dihydroazulene, to an azasugar, the bioactive component, enabling local activation at the pathological site to minimize off-target effects. The inhibitors exhibit distinct behaviors before and after light irradiation (Fig. 21). AZB, for example, can switch between its *trans* and *cis* forms under specific wavelengths, influencing the inhibitor's conformation and potency. Notably, compound **138** (Fig. 21) showed the desired light-responsive inhibition pattern, with a higher IC_{50} in the dark ($IC_{50\text{-dark}} = 24.0 \pm 3.0 \mu M$) compared to its light-irradiated form ($IC_{50\text{-light}} = 14.0 \pm 2.0 \mu M$), making it a promising candidate for photoswitchable GCCase inhibition.

Conversely, the inhibitory potency of compound **137** decreased upon light exposure, underscoring the significance of molecular design.



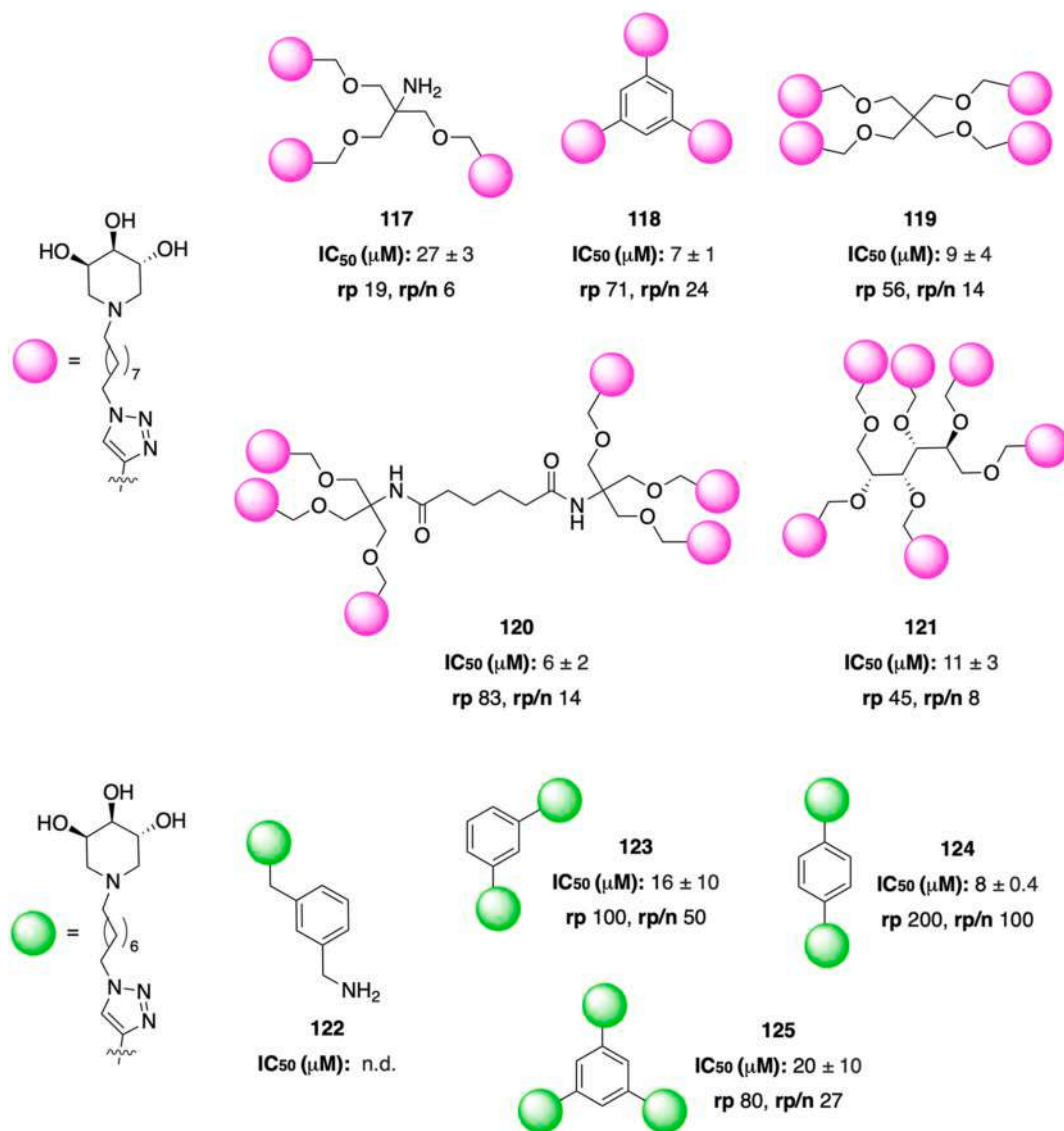


Fig. 19. Chemical structure of multivalent piperidine-based iminosugars.

Among divalent compounds, elongating the carbon chain between the iminosugar and AZB enhanced inhibitory effects, as seen with compound **140**, which demonstrated a 10-fold increase in potency after light activation ($IC_{50}\text{-light} = 3.0 \pm 0.9 \mu\text{M}$) compared to compound **139**.

To date, only one example of GCase inhibitors utilizing a multivalent approach with AuNPs as a scaffold has been reported in the literature [182]. Glyco-AuNPs are hybrid nanoparticles consisting of an ultrasmall gold core (around 2 nm in diameter) and a three-dimensional multivalent carbohydrate shell [183]. These water-dispersible, biocompatible nanoparticles have a spherical shape and a chemically well-defined composition. They are synthesized by a one-step procedure that enables precise incorporation of thiol-ending sugar ligands alongside other ligands on the same gold core [184]. Depending on the linker and headgroup, each sugar-AuNP can accommodate up to 100 ligands, allowing for optimal valency control and presentation of the bioactive iminosugar moiety for enhanced molecular recognition of target proteins [183,185,186].

Five novel AuNP-based multivalent iminosugars have been synthesized to modulate iminosugar presentation through a linear configuration (compound **141**, Fig. 22) or a dendritic trimeric arrangement (compound **142**, Fig. 22) [182]. The IC_{50} values of both compounds were comparable ($2.67 \mu\text{M}$ for **141** and $1.72 \mu\text{M}$ for **142**) when tested

against GCase obtained from leukocyte extracts. This similarity in IC_{50} values indicates a low probability of off-target interactions, reinforcing the specificity of these nanoparticles for the target. Moreover, the relative potency per nanoparticle (rp/n) values were significantly higher than 1 (17 for **141** and 13 for **142**), demonstrating a positive multivalent effect [182]. These findings strongly indicate that AuNP-dendrimer combinations significantly improve the overall efficacy of the system.

3.3. Other sugar mimetics

Due to their structural diversity and biological relevance, carbohydrate-based compounds have become a key target in developing new therapeutic agents. Iminoalditols, as previously discussed, represent one of the most widely studied classes for their potential as GCase inhibitors. Other sugar mimetics include polyhydroxylated bicyclic isoureas and guanidines [187], aminocyclitol- [188] and N-glycosides sulfanyloxazoline or azoline [189,190].

Novel aminocyclitol-based compounds with six-membered ring scaffolds have been developed over the years, demonstrating high efficacy as GCase inhibitors. Starting from compound **143** (Fig. 23), structural optimizations, such as introducing alkyl chains on both the nitrogen atom and the adjacent hydroxyl group, as well as inverting the

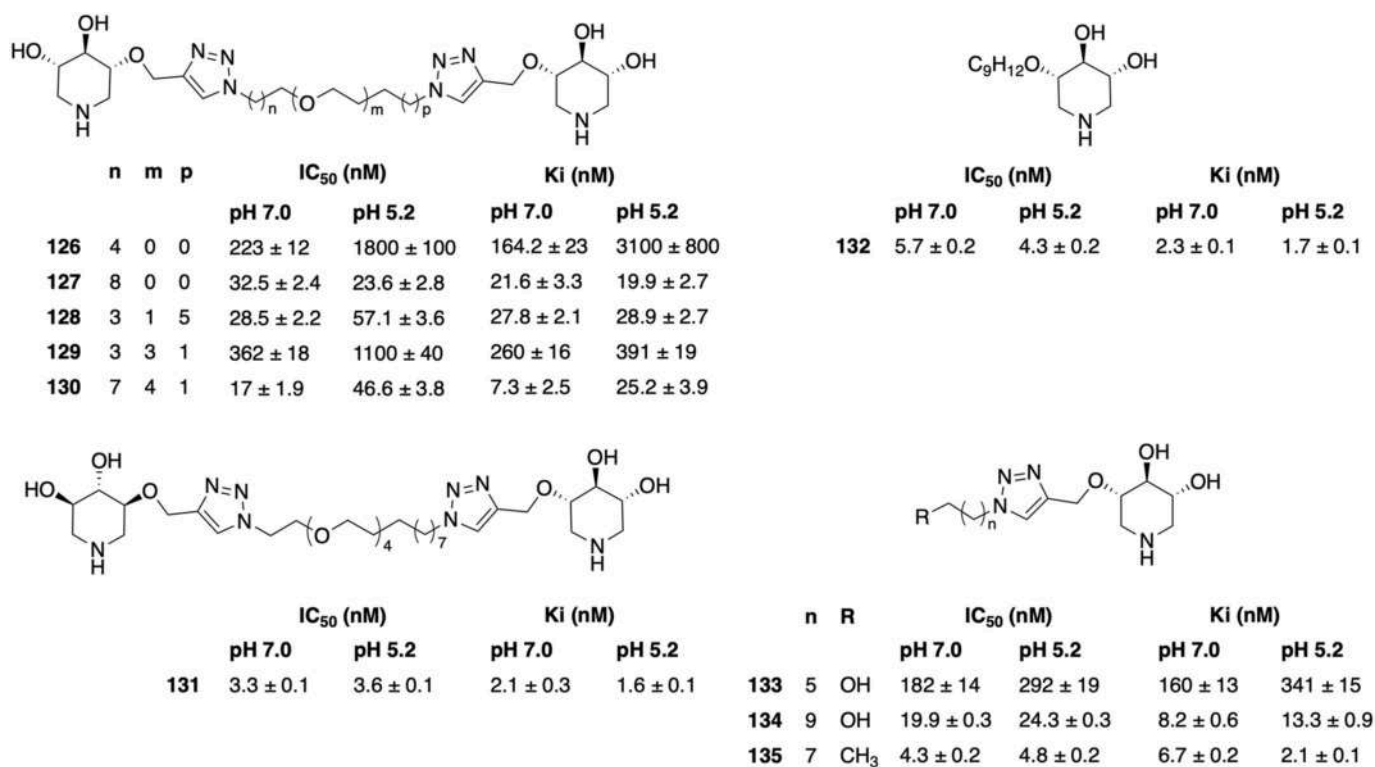


Fig. 20. Chemical structure, IC₅₀ and Ki values towards rhGCase at different pH of DIX-derived heterodimers, homodimers, and related multivalent compounds.

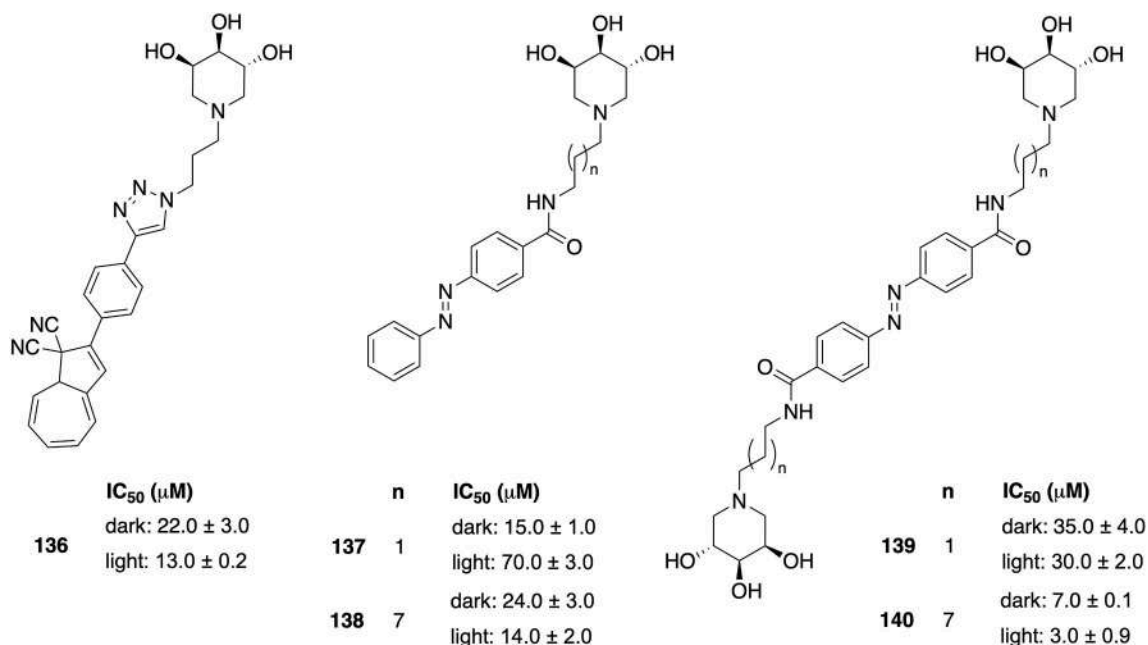


Fig. 21. Chemical structure and inhibitory potency of monovalent and divalent iminosugar-based photoswitches in dark and light-activated forms.

configuration of the stereogenic center at the C-2 position bearing a hydroxyl group, resulted in insignificant improvements in inhibitory efficiency [191]. Substitution of the hydroxyl group at the C-2 position with methyl or nonyl ether groups yielded compounds 144 and 145, respectively, where only the latter substitution proved effective. Conversely, inverting the stereogenic center at the C-2 position improved inhibitory efficiency for both methyl- and nonyl-substituted derivatives (147 and 148, respectively, Fig. 23). The free amine, resulting from the loss of the alkyl chain from the nitrogen position

(149, Fig. 23), was less active, indicating that an N-alkyl chain is required for optimal rhGCase inhibition [191]. More recently, Weber et al. screened a series of novel N-methylated aminocyclopentanes (150, 151, and 152, Fig. 23) to evaluate their glucosidases inhibition ability [192]. All compounds demonstrated potent inhibitory activity and high specificity against GCase. As previously observed, N-substitution with alkyl chains of varying lengths increased potency against the target enzyme, resulting in a decrease in Ki (Fig. 23) [192]. No inhibition of recombinant lysosomal α-D-galactosidase or human lysosomal acid

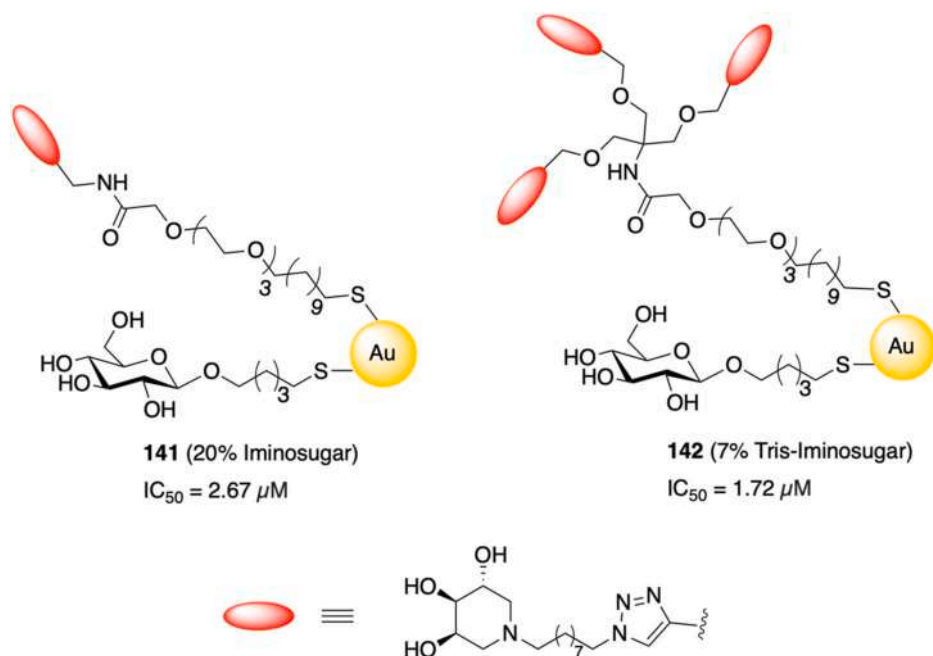
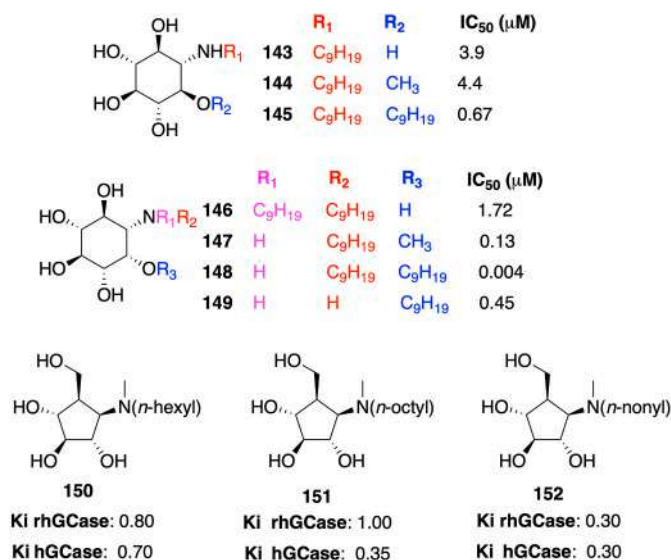


Fig. 22. Chemical structures of GCase inhibitors utilizing AuNPs as scaffolds.

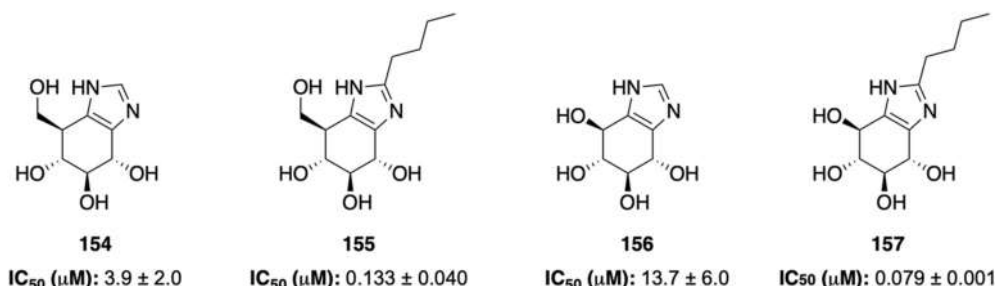
Fig. 23. Chemical structure and rhGCase/hGCase inhibition (IC₅₀/K_i, nM) of aminocyclohexanes and aminocyclopentanes derivatives.

β-D-galactosidase was observed at a 1 mM concentration, indicating selective human GCase inhibition.

Schröder et al. described the synthesis of a new class of azole-type competitive GCase inhibitors, namely gluco-1*H*-azoles [190]. An *n*-butyl chain was introduced onto the 1*H*-imidazole ring of **154**, leading to **155**, while elimination of the sugar mimetic's methylene group yielded compounds **156** and **157** (Fig. 24). These modifications resulted in potent competitive GCase inhibitors that exhibited selectivity for GCase over GBA2 (IC₅₀ > 50 μM) and human acid α-glucosidase (IC₅₀ > 100 μM), highlighting their potential as therapeutic candidates [190]. This selectivity is particularly significant because GCase over-expression is implicated in various cancers, and the ability to discriminate between lysosomal and cytosolic β-glucosidases, or between β- and α-glucosidases, could offer advantages in targeted therapies.

4. Pharmacological inhibition of GCase: non-carbohydrate-based inhibitors

In addition to carbohydrate-based inhibitors, which are well-established GCase inhibitors, several non-carbohydrate-based inhibitors have also been reported. Quantitative high-throughput screening is commonly used to rapidly identify potent and selective non-sugar GCase inhibitors. This has led to the discovery of new nanomolar GCase inhibitors with high inhibitory potency, featuring aminoquinoline, triazine, thiourea, hydroxidiazole (MLS000674724), and sulfonamide scaffolds [193–195]. These alternative inhibitors can

Fig. 24. Chemical structure and IC₅₀ values against acid lysosomal GCase of gluco-1*H*-azoles as sugar mimetics.

interact with the enzyme through non-glycosidic mechanisms, broadening the chemical space for designing potent GCase inhibitors. The development of such non-carbohydrate-based compounds is of particular interest as they can offer improved selectivity, stability, and pharmacokinetic properties, making them promising therapeutic candidates. The quinazoline ring has previously been identified as a promising scaffold for GCase inhibitor synthesis. Based on this, Zheng et al. [196] conducted a study where they introduced substituents at the 4-position of the *N*-cyclohexyl ring of compound **158** (Fig. 25) to assess their effects on GCase inhibition enhancement. Their results revealed that substitution with a smaller hydrophobic group (i.e. 4-methyl, **159**) led to a 3-fold increase in activity compared to longer groups (i.e. 4-ethyl, **160**), which produced no change in activity. This led to the synthesis of *trans*-4-methyl compound **161**, which showed a marked improvement in the inhibitory activity (IC₅₀ 20 nM). However, further modifications at the same position, such as incorporating 4-methoxyl (**162**), 4,4-dimethyl (**163**), or 4,4-difluoro (**164**), decreased potency or proved detrimental, resulting in inactive inhibitors. Next, the authors explored the possibility

of replacing the cyclohexyl ring with saturated carbon rings of various sizes (**165-169**), observing that larger cycloalkyl rings resulted in greater inhibitory potency. However, introducing more complex structures or a phenyl ring with linkers of different lengths (**174-179**), did not significantly improve activity. To enhance compound binding affinity towards GCaSe, a new series of substitutions was designed by fusing the cycloalkyl groups with a phenyl ring (**180-185**), reaching nanomolar GCaSe inhibitory activity. Finally, examining the effect of substituents at various positions of the 3-pyridine ring revealed that inhibitory potency against GCaSe consistently remained in the nanomolar range, with values comparable to or greater than those observed for **180-182** [196].

Additionally, the authors investigated the effect of different substituents on the pyridine ring, starting with compound **182**, the most potent compound they had obtained. It was demonstrated that only substituting the 3-pyridinyl group in **182** with phenyl (**186**, Fig. 25) or 3-thienyl (**187**, Fig. 25) groups resulted in compounds that retained the same potency as **182**. This finding suggests that additional groups could potentially be introduced at position 2 of the quinazoline ring. The most

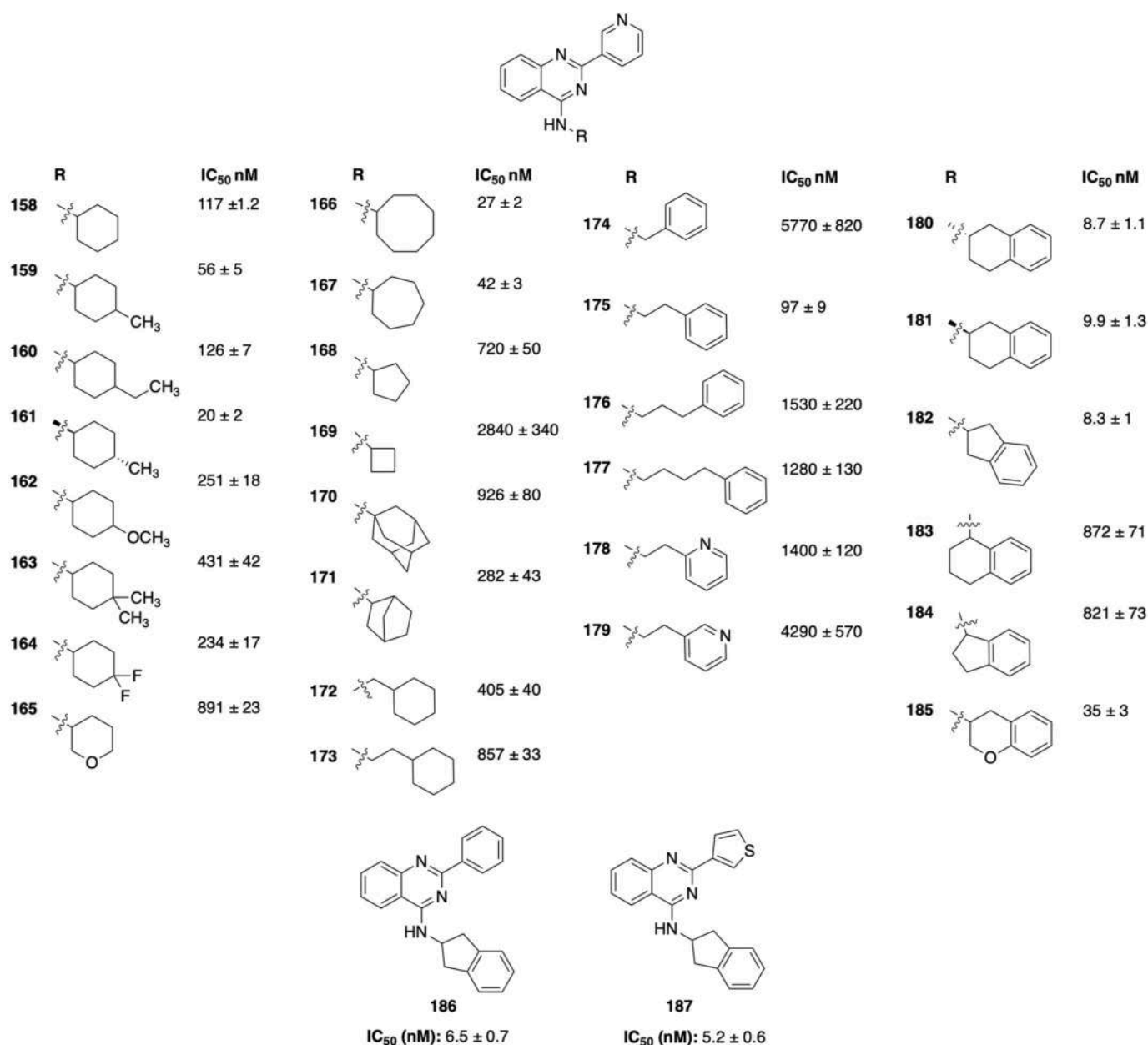


Fig. 25. Chemical structure and inhibitory potency of quinazoline-based GCaSe inhibitors.

potent compounds (**180**, **181**, **182**, and **187**) were evaluated against two additional lysosomal enzymes, namely acid α -glucosidase and α -galactosidase A. The activities of these enzymes remained largely unaltered by compound treatments at concentrations up to 100 μ M, demonstrating notable selectivity towards rhGCase over other lysosomal hydrolases [196]. Tetrahydroquinazolines have been recognized for their inhibitory properties against α - and β -glucosidases. However, initially synthesized derivatives of these molecules did not provide significant benefits in GCase inhibition [197]. Recently, modifying the 5,6,7,8-tetrahydroquinazoline structure through α -aminoamidines led to a new series of compounds, as reported by Snizhko et al. (Fig. 26) [198]. These new derivatives were found to be more active than natural GCase inhibitors. In particular, molecular docking studies on GCase from raucaffricin showed that natural inhibitors, such as quercetin and epigallocatechin, exhibit binding scores 1–2 kcal/mol lower than those of the synthesized compounds. This suggests that the new compounds could have higher binding affinity and regioselectivity towards GCase. These results highlight the potential of these molecules as new chemical scaffolds for developing highly selective and potent non-sugar inhibitors, opening new avenues in targeted therapies [198].

The synthesis of a series of novel 2-thio-4-quinazolinone derivatives was recently reported [199]. Although these compounds exhibited inhibitory potency below 10 μ M against GCase, their design was specifically oriented to achieve dual inhibition of both α - and β -glucosidase, rather than the maximization of specific efficacy against either individual enzyme.

In search for improved GCase inhibitors, a novel group of *N,N*-diarylsulfonyl and *N*-arylsulfonyl histamine derivatives was synthesized [200]. Biological investigations demonstrated that GCase inhibition occurred within the micromolar range (65–250 μ M), with *N*-4-fluorobenzenesulfonyl histamine being the most potent. Nevertheless, despite the low inhibition, this reversible and competitive inhibitor showed remarkable selectivity for GCase over other target enzymes, including α -glucosidase [200]. Finally, very recently Hoshikawa synthesized a library of pyrimidinyl piperazines, among which three compounds, **190**–**192**, exhibited rhGCase inhibition in the low micromolar range (Fig. 27) [201].

Flavonoids, naturally occurring polyphenolic compounds, are known for a wide range of biological activities, including antioxidant [202, 203], anti-inflammatory [204,205], anti-cancer [206,207] and enzyme-inhibiting effects [208]. Among them, 3-benzyloxyflavones have attracted attention due to their benzyloxy group in position 3, which increases their hydrophobicity, improves their pharmacokinetic

properties and enhances their interactions with enzymatic active sites, such as that of GCase. Several synthesized derivatives demonstrated significant inhibitory activity (Fig. 28) [209]. The compounds in this series displayed comparable inhibitory potency toward GCase with IC_{50} values ranging from 0.17 to 0.51 μ M for all derivatives except compound **193** (IC_{50} = 1.02 μ M). Compounds **199** (K_i = 90 μ M) and **200** (K_i = 75 μ M) were characterized as competitive inhibitors. Overall, relatively small differences in IC_{50} values indicate a narrow activity range of inhibitory potency across this series.

5. Conclusions and future perspectives

GCase plays a fundamental role in carbohydrate metabolism and glycoside hydrolysis and it has emerged as a key target for cancer therapy due to its dysregulation in cancer pathogenesis. In this regard, the exploration of GCase inhibition as a therapeutic coadjuvant in cancer treatment is highly promising. Early studies using genetic and pharmacological strategies to inhibit GCase, in co-administration with chemotherapeutic agents, have shown encouraging results. Unfortunately, there are currently no clinical trials investigating the co-administration of GCase inhibitors with chemotherapeutic agents to evaluate potential enhancements in chemotherapy efficacy. While pre-clinical studies have shown promise in combining GCase inhibitors with other cancer therapies, this approach has yet to be explored clinically. Following the promising results of CBE, the more selective GCase inhibitors discussed in this Review could represent promising candidates for testing in various cancer models. In particular, blocking the enzyme can interfere with tumor cell proliferation and increase apoptosis. Therefore, the synergistic potential of GCase inhibitors, when combined with other therapeutic agents, may offer new strategies to enhance the efficacy of existing cancer treatments. Several sugar mimetics and non-carbohydrate-based inhibitors have demonstrated potent competitive and non-competitive inhibitory capabilities in the nanomolar range. Structural modifications play a critical role in improving inhibitor selectivity and potency. Successful modifications include lengthening and/or repositioning alkyl chains and selecting specific enantiomers with higher affinity for the enzyme's active site. Additionally, the introduction of aliphatic and aromatic rings with strategically placed substituents further improves the interaction with the enzyme through allosteric binding.

While the development of GCase inhibitors has traditionally relied on the synthetic diversification of known carbohydrate mimics (e.g., iminosugars, aminocyclitols), emerging computational methodologies offer a transformative paradigm for discovering novel chemical entities and rationalizing structural selectivity. Given the challenges associated with the off-target inhibition of ubiquitous glycosidases like GBA2 and GBA3, the integration of structure-based virtual screening (SBVS) and molecular dynamics (MD) simulations represents a crucial frontier in the design of next-generation cancer theranostics and chemical probes. The large number of available X-ray crystal structures of human GBA1 (GH30), including both the apo enzyme and complexes with a variety of inhibitors (e.g., CBE, isofagomine, and ambroxol), provides a robust structural framework for SBVS campaigns.

High-throughput docking of diverse, lead-like chemical libraries (such as ZINC or Enamine) into the deep, canonical (β/α)₈ TIM-barrel active site of GBA1 allows for the identification of entirely non-carbohydrate, topologically unique scaffolds. Beyond the rigid catalytic pocket defined by Glu340 and Glu235, SBVS can be directed toward the distinct, narrow hydrophobic aglycone-binding channel of GBA1, or newly identified allosteric sites. This strategy is particularly valuable for discovering non-competitive pharmacological chaperones or inhibitors that lack the highly polar, polyhydroxylated features of traditional iminosugars, thereby improving overall drug-like properties, metabolic stability, and membrane permeability in cellular cancer models.

Static crystallographic data often fails to capture the structural

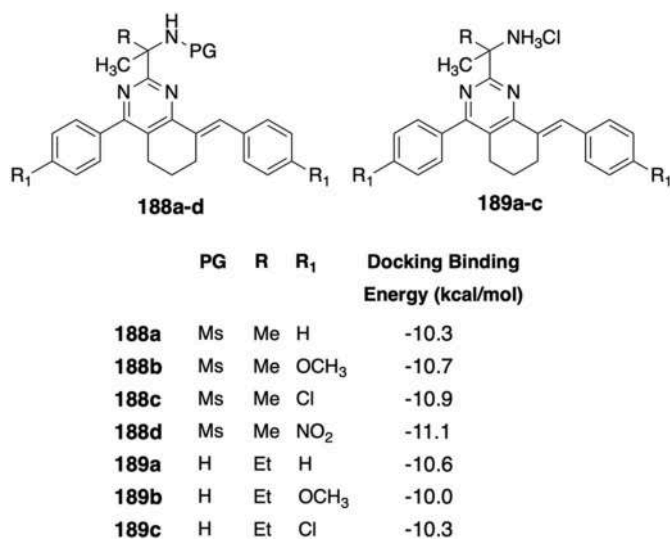


Fig. 26. Chemical structure and molecular docking binding score of tetrahydroquinazoline derivatives as GCase inhibitors.

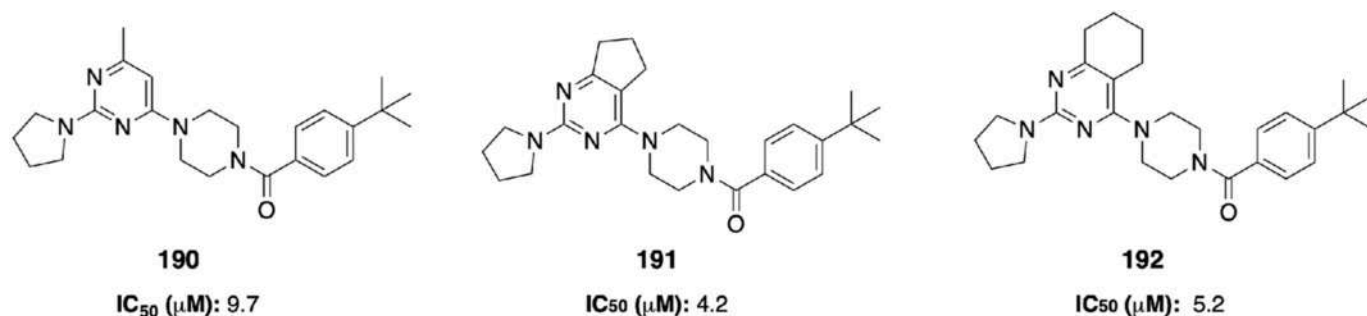
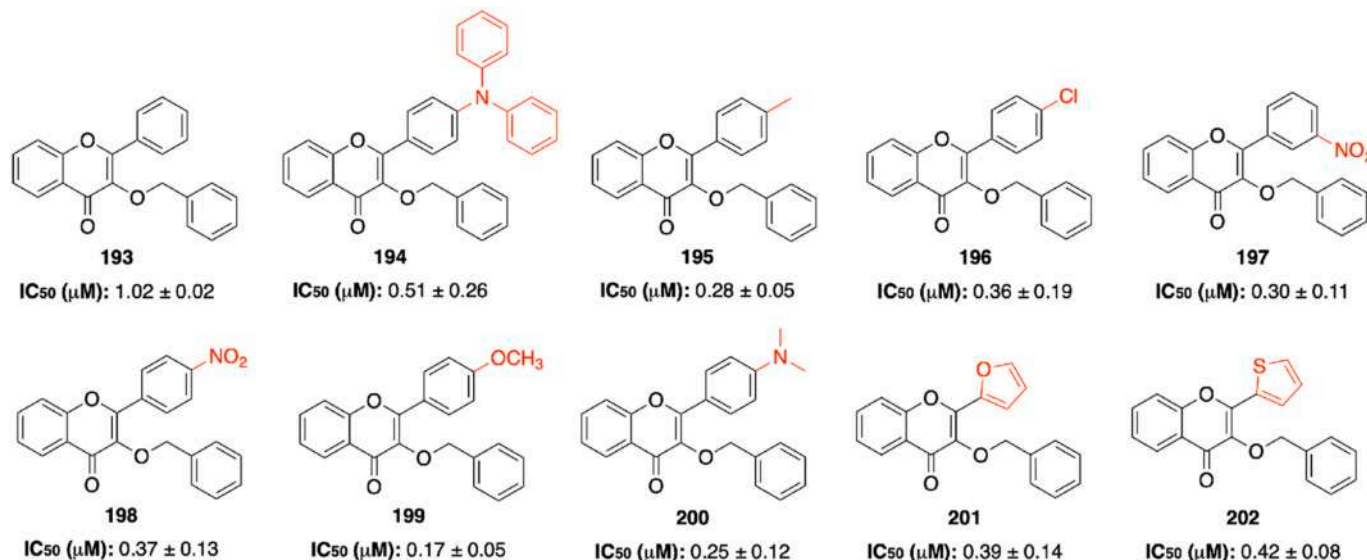


Fig. 27. Chemical structure and GCase inhibition values of some pyrimidinyl piperazines.

Fig. 28. Chemical structure and IC_{50} GCase inhibition values of 3-benzoyloxyflavone derivatives.

variations that dictate enzyme function and ligand recognition. Classical and enhanced-sampling MD simulations provide critical insights into the dynamical behavior of these glycosidases. In GBA1, active-site accessibility is modulated by highly flexible loop regions (specifically Loops 1, 2, and 3) that undergo significant conformational rearrangements upon ligand binding. MD simulations can trace these transitions, allowing researchers to design “conformation-specific” inhibitors that lock the enzyme in a particular inactive or active state. Utilizing techniques such as Umbrella Sampling, Metadynamics, or Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) calculations allow for the precise estimation of binding free energies (ΔG_{bind}). This provides a quantitative atomistic breakdown of the specific electrostatic, hydrophobic, and van der Waals interactions driving ligand stabilization.

The primary hurdle in GCase drug design remains the cross-reactivity with the non-lysosomal β -glucosidases GBA2 (GH116) and GBA3 (GH1). Because these enzymes share no significant primary sequence homology and fold into fundamentally distinct structural architectures, comparative MD simulations offer an unparalleled tool to drive rational design. While GBA1 features a deep, sterically restricted pocket, homologous or experimental models of GBA2 demonstrate a much wider, more solvent-exposed active site. MD simulations can monitor how a simulated ligand interacts within these contrasting environments, revealing distinct water-network dynamics and localized desolvation energies. By comparing the trajectories of a candidate inhibitor across GBA1, GBA2, and GBA3 simultaneously, researchers can map out “selectivity hot-spots” (specific amino acid residues unique to the GBA1 binding cleft that do not align or match spatially in GBA2 or GBA3). Designing molecules with specific functional groups tailored to form salt bridges or π - π

stacking interactions exclusively with these GBA1-specific residues may provide a clear path toward eliminating off-target side effects. Ultimately, combining iterative machine learning-driven virtual screenings with atomistic MD trajectories will significantly reduce the costly, trial-and-error synthetic cycles typically required to achieve isoform-selective glycosidase inhibition, paving the way for highly precise therapeutic interventions.

Overall, further investigation is essential to fully exploit the therapeutic potential of GCase inhibition in cancer. Understanding the precise mechanisms by which these inhibitors interact with GCase and how they influence cellular pathways is crucial. This knowledge could clarify the extent to which GCase inhibition can synergistically enhance the cytotoxic effects of chemotherapeutics while avoiding unwanted cellular dysregulation or toxicity. Furthermore, the discovery and development of GCase inhibitors with increased potency and selectivity are key goals. Advances in structure-activity relationship studies and in computational modelling offer promising avenues to identify and optimize these inhibitors.

CRediT authorship contribution statement

Francesca Milano: Writing – review & editing, Writing – original draft, Visualization, Data curation, Conceptualization. **Francesca Clemente:** Writing – review & editing, Visualization, Data curation, Conceptualization. **Francesca Cardona:** Writing – review & editing, Supervision. **Andrea Goti:** Writing – review & editing, Supervision, Funding acquisition. **Alessandra Quarta:** Writing – review & editing, Supervision. **Marco Marradi:** Writing – review & editing, Supervision,

Funding acquisition. **Andrea Ragusa:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Camilla Matassini:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Funding sources

Financial support provided by MUR – Dipartimenti di Eccellenza 2023-2027 (DICUS 2.0, CUP: B97G22000740001) to the Department of Chemistry “Ugo Schiff” of the University of Florence is acknowledged. This research was supported by “Tecnopolo per la medicina di precisione” (TecnoMed Puglia)–Regione Puglia: DGR n.2117 del 21/11/2018 (CUP: B84I18000540002), and “Tecnopolo di Nanotecnologia e Fotonica per la medicina di precisione” (TECNOMED)–FISR/MIUR-CNR: delibera CIPE n. 3449 del 7–08–2017 (CUP: B83B17000010001). A.G., F.M. and M.M. thank for co-funding the European Union NextGeneration EU programme in the context of the National Recovery and Resilience Plan, Mission 4, Component 2, Investment 1.5, ECS00000017, THE “Tuscany Health Ecosystem” – Spoke 4 “Nanotechnologies for diagnosis and therapy”, CUP: B83C22003920001.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

4MU- α -D-gal	4-Methylumbelliferyl- α -D-galactopyranoside
4MU- α -D-glc	4-Methylumbelliferyl- α -D-glucopyranoside
4MU- β -D-gal	4-Methylumbelliferyl- β -D-galactopyranoside
4MU- β -D-glc	4-Methylumbelliferyl- β -D-glucopyranoside
5-FU	5-Fluorouracil
ABPs	Activity-based probes
AMP-DNM	N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin
AuNPs	gold nanoparticles
AZB	Azobenzene
C1P	Ceramide 1-phosphate
CAzy	Carbohydrate-Active Enzymes
CBE	Conduritol β -epoxide
CP	Cyclophellitol
CuAAC	Copper(I)-Catalyzed Azide-Alkyne Cycloaddition
DAB	1,4-dideoxy-1,4-imino-D-arabinitol
DIX	1,5-dideoxy-1,5-imino-D-xylitol
DLB	dementia with Lewy bodies
DNJ	1-deoxynojirimycin
DNP- α -gal	2,4-Dinitrophenyl- α -D-galactopyranoside
DOX	Doxorubicin
EMT	Epithelial-Mesenchymal Transition
GAA	recombinant human α -glucosidase
GALC	β -galactocerebrosidase
GBA	β -glucocerebrosidase
GCase	β -glucocerebrosidase
GEM	Gemcitabine
GEPIA	Gene Expression Profiling Interactive Analysis
GH	Glycoside Hydrolase
GlcCer	glucosylceramide
GSLs	Glycosphingolipids
HCC	Hepatocellular carcinoma
IC ₅₀	half-maximal inhibitory concentration
IFG	Isofagomine
IHC	Immunohistochemistry
LAB	1,4-dideoxy-1,4-imino-L-arabinitol
LacCer	Lactosylceramides
LSDs	Lysosomal Storage Diseases

Lyso-Gb1	glucosylsphingosine
MD	molecular dynamics
MET	Mesenchymal-Epithelial Transition
MM-PBSA	Molecular Mechanics Poisson Boltzmann Surface Area
monoHex-Cer	Monohexosylceramides
n	number of binding units
NN-DNJ	N-nonyldeoxynojirimycin
p-AXL	phosphorylated AXL
PAM	PI3K/Akt/mTOR (Phosphoinositide 3-kinase/Protein kinase B/Mammalian target of rapamycin)
PCs	Pharmacological Chaperones
pNP- α -D-glc	4-Nitrophenyl- α -D-glucopyranoside
pNP- β -D-gal	4-Nitrophenyl- β -D-galactopyranoside
PTX	Paclitaxel
RDA	reductive amination approach
rhGCase	recombinant human GCase
ROS	Reactive Oxygen Species
rp	relative potency
RTK-AXL	AXL Receptor Tyrosine Kinase
SBVS	Structure-Based Virtual Screening
siRNA	small interfering RNA
TxGH116	Thermoanaerobacterium xylanolyticum GH116
α -glu	α -glucosidase
β -gal	β -galactosidase

Data availability

No new data were created or analyzed in this study (Review paper).

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